Universidade de Aveiro Departamento de Biologia
2017

Emanuele Fasola

Aquisição e hereditariedade de tolerância a metais em anuros.

Acquisition and inheritance of tolerance to metals in anurans.

Universidade de Aveiro Departamento de Biologia
2017

Emanuele Fasola

Aquisição e hereditariedade de tolerância a metais em anuros.

Acquisition and inheritance of tolerance to metals in anurans.

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutoramento em Biologia, realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes (Investigadora Principal do CESAM e Departamento de Biologia da Universidade de Aveiro), do Professor Doutor Rui Godinho Lobo Girão Ribeiro (Professor Associado com Agregação do Departamento de Ciências da Vida da Universidade de Coimbra) e da Professora Doutora Paula Maria de Melim e Vasconcelos de Vitorino Morais (Professora Auxiliar com nomeação definitiva no Departamento de Ciências da Vida da Universidade de Coimbra).

This work was supported by FEDER funds within the PT2020 Partnership Agreement and Compete 2020- Programa Operacional Factores de Competitividade, by the Portuguese Foundation for Science and Technology (FCT), and by the research project GENEROSI PTDC/BIA-BIC/3488/2012, within the CESAM's strategic programme (UID/AMB/50017/2013), this work was also funded by national funds via FCT/MEC (PIDDAC) and by the doctoral fellowship of Emanuele Fasola SFRH/BD/88955/2012.

Dedico il mio dottorato ai miei genitori, che mi hanno dato fiducia e appoggio nonostante le situazioni avverse che si incontrano nel cammino della vita.

o júri

Presidente:

Prof. Doutor Rui Luís Andrade Aguiar
Professor catedrático do Departamento de Eletrónica, Telecomunicações e Informática
da Universidade de Aveiro

Vogais:

Doutora Isabel Maria Cunha Antunes Lopes
Investigadora Principal do CESAM e Departamento de Biologia
da Universidade de Aveiro (Orientadora)

Prof. Doutor Miguel Alberto Fernandes Machado e Santos
Professor auxiliar da Faculdade de Ciências
da Universidade do Porto

Doutora Isabel da Silva Henriques
Equiparada a investigadora auxiliar do Departamento de Biologia
da Universidade de Aveiro

Doutor Manuel Eloy Ortiz-Santaliestra
Investigador do Instituto de Investigación en recursos Cinégeticos
da Universidad de Castilla-La Mancha

Doutora Matilde Maria Moreira dos Santos
Investigadora em Pós-doutoramento Da Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

agradecimentos

À minha orientadora Dra. Isabel Lopes, por todo o apoio científico, e por ser muitos mais do que uma orientadora. Alem disso um ponto firmo de ajuda e compreensão quando encontrei uns graves problemas ao longo do caminho. Ao professor Rui Ribeiro por todas as ideias, que a sua mente vulcânica mal consegue conter, não podia desejar melhor mentor cientifico. A professora Paula Morais pela grande ajuda, competência e serenidade com as quais sempre trabalhou comigo.

A todos os colegas que me ajudar durante o trabalho, seja de campo ou de laboratório: Diogo, Barbara, Sara C., Sara P., Cátia, Bruno, Antonieta, Anabela, Nuno C., Nuno M., Inês, Jorge, Ana, Tânia M., Catarina, Miguel, Tânia D., Marta e Carla. Aos espanhóis e italianos que fizerem parte desta viagem: Manuel, Rafa, Claudia e Marta.

Ao Abel, o melhor técnico do mundo (e não por ser o único) mas por ser único!

A toda a gente do laboratório, porque foram ótimos colegas, mas sobre todos amigos dentro e fora do trabalho. A toda a gente do basket e do magic, vocês são demasiados para eu poder escrever os vossos nomes, mas obrigado pela vossa amizade e para tudo!

palavras-chave

Pelophylax perezi, variabilidade genética, plasticidade ambiental, dominância genética incompleta, contaminação por metais, erosão genética, mecanismos de hereditariedade da tolerância, microbioma da pele.

resumo

Os anfíbios encontram-se em declínio a nível global, sendo a contaminação química um dos principais fatores associados a este declínio. De facto, a exposição de populações de anfíbios a este tipo de perturbações ambientais pode provocar a perda de diversidade genética, devido à diminuição de: 1) aptidão, 2) plasticidade ambiental e 3) eficácia dos mecanismos de tolerância.

A contaminação por metais é uma das causas de poluição mais comum no mundo, estando presente em larga escala na Península Ibérica, nomeadamente na Faixa Piritosa Ibérica. Deste modo o estudo dos mecanismos de tolerância a metais, em anfíbios, é relevante, assim como a investigação sobre os mecanismos de hereditariedade desta tolerância. Um dos objetivos do presente trabalho centrou-se no estudo da hereditariedade de tolerância a metais em ovos de *Pelophylax perezi*. Os resultados obtidos sugerem uma dominância genética incompleta como sendo o mecanismo mais provável de hereditariedade de tolerância a contaminação por metais, em ovos de *P. perezi*. Estes resultados suportam a hipótese de hereditariedade de tolerância recessiva (ou dominância incompleta). Neste contexto, uma perda de diversidade genética em populações de anfíbios, expostas a contaminação por metais pode ocorrer, mesmo que a fixação de alelos na população seja excluída.

De modo a estudar a possibilidade de girinos adquirirem maior tolerância a contaminação por metais, devido à sua exposição histórica a este tipo de contaminação, foram recolhidos girinos de *P. perezi* em locais contaminados por metais e em locais de referência. Posteriormente, os girinos foram expostos, em laboratório, a um pulso de contaminação intensa por metais. Os girinos oriundos de locais contaminados não mostraram maior tolerância à toxicidade letal de metais comparativamente aos girinos recolhidos em locais de referência. Mais ainda, não revelaram estar sujeitos a um maior stress oxidativo. No entanto, a quantidade de metais no corpo provou a contaminação por metais nos locais historicamente impactados e mostrou que os iões de mercúrio e chumbo são prontamente biodisponíveis para os girinos de *P. perezi*. Os girinos de locais contaminados apresentaram níveis constitutivos de metalotioneínas, superiores aos medidos nos girinos recolhido os nos locais de referência, o que pode indicar adaptação a contaminação por metais. Por fim, o último objetivo consistiu em avaliar a influência de contaminação química na composição e diversidade do microbioma da pele de populações de *P. perezi* bem como, identificar a sua sensibilidade a contaminação por efluentes de drenagem ácida. O microbioma da pele dos anfíbios apresenta um papel fundamental na proteção destes organismos a agentes perturbadores ambientais.

Os resultados obtidos revelaram que os metais podem influenciar a composição da comunidade microbiana de anfíbios que habitam locais contaminados. Mais ainda, uma concentração elevada de efluente de drenagem mineira inibiu o crescimento da maioria dos isolados de bactérias da pele dos anfíbios. Esta inibição pode sugerir que os anfíbios perdem uma fração importante do seu microbioma e consequentemente, afetar a proteção da sua pele, quando expostos a contaminação por metais, o que pode determinar um aumento da sensibilidade a este tipo de contaminação.

keywords

Pelophylax perezi, genetic variability, environmental plasticity, incomplete dominance, metal contamination, genetic erosion, tolerance inheritance, skin microbiome.

abstract

Amphibians are declining globally, chemical contamination being one of the major factors driving this process. As a consequence of exposure to such environmental perturbation, natural population of amphibians may lose their genetic diversity, which may occur due to a decrease in: 1) fitness, 2) environmental plasticity capabilities and 3) tolerance mechanisms efficiency. Metal contamination is one of the most worldwide distributed contamination source, having a great impact in the Iberian Peninsula habitats, especially in the Iberian pyrite belt region. Therefore, is important to explore how tolerance mechanism, toward metal contamination, work in amphibians and how genetically determined tolerance mechanisms are inherited. In this work, these topics were addressed by assessing the inheritance to lethal tolerance to acid mine drainage and copper contamination in eggs of the Perez's frog *Pelophylax perezi*. Incomplete dominance was found to be the most likely inheritance mechanism of tolerance toward these two chemical stressors in the eggs of *P. perezi*. The results support the recessive (or incompletely dominant) tolerance inheritance (working-) hypothesis. Thus, the amphibians' populations impacted by metal contamination can considerably lower their genetic diversity, even if allele fixation was excluded.

The possibility of tadpoles, historically exposed to metal contamination, being able to acquire an increased tolerance to metal contamination, comparatively to tadpoles inhabiting reference sites, was also studied. *Pelophylax perezi* tadpoles, sampled at historically metal impacted mining sites, did not show higher oxidative stress or lethal tolerance comparatively to tadpoles inhabiting reference sites. However, the metal body burden proved metal contamination at the historically metal impacted sites and showed that mercury and lead ions are readily bioavailable for *P. perezi* tadpoles. Furthermore, tadpoles from metal contaminated sites seem to show higher constitutive levels of metallothioneins, which may suggest adaptation to metal contamination

The last objective of this work, was to evaluate the influence, of metal contamination, on the composition and diversity of the *P. perezi* skin microbiome, and to explore its tolerance to acid mine drainage contamination. Amphibians' skin microbial community has been shown to help its hosts tolerating infections. Because the increasing research on the important protective role of amphibians' skin microbiome, its diversity and capacity to tolerate metal contamination was as well investigated. Obtained results showed that metal contamination influences the skin microbial community composition in frogs living at metal impacted sites; furthermore, an intense acid mine drainage concentration can inhibit the growth of almost all the isolated strains. This inhibition suggests that amphibians may lose an important part of their skin microbiome, affecting the protection of their skin, when exposed to metal contamination; which, in turn may lead to an increased sensitivity to metal contamination.

Index

Index of figures

CHAPTER 1.

General Introduction.

General introduction.

Brief overview on biodiversity

Biodiversity has been perceived and investigated in many different ways, as its main components have many times been questioned (Mace *et al.* 2012). The concept has been firstly defined in the United Nations Conference on Environment and Development, held in Rio de Janeiro in 1992 (Rio Convention, 1992). Biodiversity is largely interpreted as a measure of species richness, with species as its core unit, both for research and conservation purposes (Purvis and Hector, 2000). The concept also comprehends spatial-geographic components, thus considering the variation in habitats and ecosystems, among climatic geographical regions (Fischer and Young, 2007; Fleishman *et al.* 2006). This facilitated the designation of hotspot biodiversity areas as important for conservation, and the recognition of particular sites deserving to be promoted as protected areas (Olson *et al.*, 2001). Indeed, the importance of species conservation is deeply connected with the preservation of the ecosystems in which they live (Mace *et al.* 2012). The concept established in the Rio convention was later officially accepted by the Parties of the Convention on Biological Diversity (CBD); the definition of biodiversity is thus given as: 'the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems' (CBD, 2013; Rio Convention, 1992). Biodiversity, as agreed at the Rio convention, has three main components: a) diversity of landscapes and ecosystems inside them, b) species diversity, and c) genetic variability between and within populations of the same species (*Agenda 21 : programme of action for sustainable development ; Rio Declaration on Environment and Development ; Statement of Forest Principles: The final text of agreements negotiated by governments at the United Nations conference on environment and devel*, 1993). These components have been regarded as the three pillars of biodiversity, each one deserving to be taken into account when planning conservation policies (CBD, 2013). That is because biodiversity supports life on planet Earth, and all around the world is possible to spot a plethora of variation in nature (Odum and Barrett, 2004; Raven and Johnson, 2014; Sala *et al.* 2000). One of the important components included in the definition of biodiversity, as one of its three pillars, is the genetic variability, also because it can ensure population capability to adapt to future environmental changes (Boyce, 1992; Van Straalen and Timmermans, 2002). Scholars categorized organisms inhabiting Earth into taxa, creating different taxa-diversity levels. Although being an artificial classification, it is useful to understand reciprocal relatedness into group of living

beings (Wiley and Leiberman, 2011). Most important, taxa divisions were fundamental for research and for conservation. A lot of studies and research projects came out with a flag-species (charismatic and symbolic species with the purpose to stimulate conservation awareness) on the spot, because it was convenient and appealing (Dietz *et al.* 1994; Entwistle and Bowen-Jones, 2002). Indeed, protecting some noticeable or conspicuous species can end up protecting all the food-web-network around it, or even all ecosystem that harbors it (Simberloff, 1998). Even if species level has been at the core of conservation, none of the biodiversity pillars is inherently more important than the others, they interact with each other and with many factors that can impact them (Via and Lande, 1985). All together, concurring in shaping biodiversity (*Agenda 21 : programme of action for sustainable development ; Rio Declaration on Environment and Development ; Statement of Forest Principles: The final text of agreements negotiated by governments at the United Nations conference on environment and devel*, 1993; Odum and Barrett, 2004). Once again, impacts on one level of biodiversity affect the others two levels with upward and downward cascades. For example, habitat fragmentation can enable speciation (Odum and Barrett, 2004). But, if the isolated population is too small, it can experience a bottleneck, lowering its genetic diversity and precipitating its possible extinction. This process is more effective, in small populations, if the gene flow is interrupted or if the level of inbreeding is high (Hedrick and Kalinowski, 2000; Keller and Waller, 2002). On the other hand, the loss of a species may impact the ecosystem functions, altering its capabilities to provide services (Mace *et al.* 2012; Medina *et al.* 2007; Vinebrooke *et al.*, 2004; Worm *et al.*, 2006). Scholars investigated the mechanism and the causes behind biodiversity loss, to understand why it occurs and how to prevent it (Miller, 2005; Myers *et al.*, 2000). Indeed, human activities produce a wide variety of adverse impacts on biodiversity. For example, urbanization enhances habitat fragmentation (Carr and Fahrig, 2001; Fahrig, 1997; McKinney, 2002; Wiegand *et al.* 2005), while agriculture and industry generate pollution by various kind of toxic compounds (Abler and Shortle, 2001; Bridges, 2000; Brühl *et al.*, 2013; Nadal *et al.* 2004). Pollution is a concerning issue since it can lower biodiversity regarding all its three fundamental components. For example, marine ecosystem structure and services may be hindered by fertilizers, pesticides, sewage sludge, and metals; these pollutants can cause direct mortality of the primary and secondary consumers (edible crustaceans and fish), impairing the fishery industry. Furthermore, these waters would not be available for recreational use anymore (Islam and Tanaka, 2004). Chemical contamination is among the main causes of species decline nowadays (Fisker *et al.*, 2011; Rouse *et al.*, 1999; Wilcove *et al.*, 1998). Moreover, contamination can impact genetic diversity directly; for example, through mutagenicity induced in plants by metal contaminated soil (Knasmüller *et al.*, 1998). Metal contamination can also have drastic effects on microbial communities; the strains in this communities can develop tolerance to

metal contamination (Gadd and Griffiths 1977; Díaz-Raviña *et al.*, 1994). Moreover, toxic compounds may cause genetic erosion (Fasola *et al.* 2015; Ribeiro and Lopes, 2013; Van Straalen and Timmermans, 2002). Therefore, it is important to enhance ecotoxicology research, because all human-generated impacts can alter nature's processes in many more subtle ways than that which is conceivable at a single biodiversity level (Relyea and Diecks, 2008).

Impact of anthropogenic contamination on genetic diversity

Genetic erosion is the loss of genetic variation: the loss of genotypes determining a specific trait or set of traits (Fasola *et al.* 2015; Van Straalen and Timmermans, 2002). When genetic erosion is due to chemical contamination, it can be a serious threat to the viability of populations, especially small ones (Coutellec and Barata, 2011; Lopes *et al.*, 2009; Luquet *et al.*, 2011; Medina *et al.* 2007; Ribeiro and Lopes, 2013). This is because the loss of genetic variation, due to contamination, may impact: 1) average fitness of the population, 2) environmental plasticity, 3) co-tolerance mechanisms, 4) trade-off mechanisms, and 5) tolerance to pathogens or diseases (Fasola *et al.* 2015). When a population is exposed to a contaminant, the loss of some of its individuals (sensitive to this particular compound), either by death or emigration, causes a reduction of its genetic variability. Genetic erosion can be caused by contaminant-driven directional selection or contaminant-driven genetic drift, in practice inducing bottleneck effects (for example through mechanism of habitat fragmentation), leading to inbreeding (Fasola *et al.* 2015; Ribeiro and Lopes, 2013). The effects of contaminant-driven genetic erosion can be worsened if this process acts on already fragmented populations, that often show high levels of inbreeding (Channell and Lomolino, 2000; Chen *et al.*, 2012; Hedrick and Kalinowski, 2000; Keller and Waller, 2002; Luquet *et al.*, 2013). Those populations may be hardly impacted by contaminant-driven genetic variability loss, and deserve special attention and dedicated conservation practices (Channell and Lomolino, 2000; Fasola *et al.* 2015). In this context, vertebrates' populations, especially those of endangered or vulnerable species, may be at higher risk of extinction (IUCN, 2013). Among them, amphibians arise major concerns about their conservation, as, at present, they are facing a global decline.

Global amphibian decline

Of the know species of amphibians in the world, 40% (about 6000 species; Pyron and Wiens, 2011), are considered to be declining, with 30% having a concerning conservation status according to the International Union for Conservation of Nature (IUCN, 2013). Most studies that have investigated

the amphibian decline point out that many populations are moving toward extinction (Alford and Richards, 2007; Blaustein and Bancroft, 2007; Blaustein *et al.* 1994; Collins and Storfer, 2003; Houlahan *et al.*, 2000; Kiesecker *et al.* 2001; Stuart *et al.* 2004). Emblematic is the case of the golden toad (*Incilius periglenes*) and the harlequin frog (*Atelopus varius*), which have been linked to pollutants or diseases (Pounds and Crump, 1994). Amphibian populations are known to be fragmented and with low gene flow, given their relatively low dispersal capabilities (Beebee, 2005; Bielby *et al.*, 2013; Smith and Green, 2005). Furthermore, those populations often present low effective sizes, increasing the probability of inbreeding and genetic erosion (Beebee, 2005; Beebee and Griffiths, 2005; Laan and Verboom, 1990). The majority of amphibians, mainly of the order Anura, go through metamorphosis, living in aquatic habitat as larvae and in the terrestrial compartment as adults (Pough and Kamel, 1984; Sparling *et al.*, 2010; Wilbur, 1980; Wilbur and Collins, 1973). For this reason, amphibians enter in contact with contaminants characteristics of both aquatic and terrestrial ecosystems (Blaustein and Bancroft, 2007), again increasing the probability for contaminant-driven genetic erosion to act on their populations (Fasola *et al.* 2015; Medina *et al.* 2007). It has already been reported that many compounds, like pesticides, and pathogens can induce lethal effects, ultimately reducing populations and possibly leading to bottlenecks and decrease of the genetic pool of such populations (Baud and Beck, 2005; Bridges, 2000; Brühl *et al.*, 2013; Corn and Vertucci, 1992; Hall and Henry, 1992; Hatch and Blaustein, 2003; Hua *et al.* 2013; Ireland, 1991; Macìas *et al.* 2007). Also, the gene flow among amphibian populations and genetic drift rates can be modified by humans' activities, which often fragment or destroy habitats suitable for amphibians (Cushman, 2006). In addition, toxic compounds, deriving from human activities, may promote diseases in amphibians, because some pollutants have the capability to impair amphibian immunity (Jobling *et al*., 2013). Moreover, if the physiological condition of an individual is already lowered by the effect of contamination (for example by slowing its metabolism), then immune responses would be reduced in magnitude and efficacy (Fedorenkova and Vonk, 2012; Folt *et al.*, 1999; Hatch and Blaustein, 2003; Mazanti *et al.*, 2003; Vinebrooke *et al.*, 2004; Wah-Chu and Chow, 2002; Yue *et al.*, 2009). Another important factor within the frame of amphibians' decline is the spread of diseases and pathogens, which are decimating many populations. For example, chytrid fungus, *Ranavirus* and water mould infections are a big threat to amphibians (Baláž *et al.*, 2013; Fernández-Benéitez *et al.*, 2011; Pearman and Garner, 2005; Pounds *et al.*, 2006; Stevenson *et al.*, 2013).

Amphibian tolerance to contamination and pathogens

Given the many adverse impacts that amphibian populations have to face nowadays, the tolerance mechanisms they possess, to several environmental perturbations, are crucial for survival. These capabilities may be due to selected genetic traits, in the population, leading to micro-evolutionary shifts in alleles frequencies (Dieckmann and Doebeli, 2004). Tolerance can also derive from phenotypic plasticity (Ghalambor *et al.*, 2007; Via *et al.* 1995; Weitere *et al.*, 2004). In either case, amphibians can face contamination and pathogens induced stress with many mechanisms. For example, Moor frogs' (*Rana arvalis*) Swedish populations rapidly evolved tolerance to water acidification (Rasanen *et al,* 2003). The authors hypothesized that such increased tolerance was due to a physiologic mechanism likely related to the osmotic regulation or to a more efficient way for egg's jelly coat to block hydrogen ions. This grants embryos and tadpoles the ability to tolerate low pH (Rasanen *et al.* 2003). Another study showed the origin, by natural selection, of osmotic stress tolerance in tadpoles of the natterjack toad (*Bufo calamita*) inhabiting brackish ponds, possibly resulting from drought tolerance by exaptation (Gomez-Mestre *et al.*, 2004). Another study pointed out wood frogs' (*Lithobates sylvaticus*) tadpoles capabilities for plastic responses toward pesticide contamination, by mean of inducible tolerance caused by previous exposure to sublethal concentrations of carbaryl (Hua *et al.* 2013). Wood frog was also an interesting case of maternal effects combined with genetically determined tolerance to acidification; the maternal effects determined embryo's tolerance, while genetic variation determined tolerance of larvae, and thus embryo's tolerance was not related with larval tolerance (Pierce and Sikand, 1985). Amphibians also harbor a rich and diverse microbial community onto their skin. This can be an effective defensive mechanism, which is not characteristic of the amphibian itself but of its skin associated microbial community. Some studies referred how their skin microbiome can contain bacteria strains tolerant to some pathogens or even pollutants (Becker *et al.*, 2009; Choi *et al.*, 2015; Díaz-Raviña et al., 1994; Dobson *et al.*, 2012; Gadd and Griffiths 1977; Harris, Brucker e Walke, 2009; Woodhams *et al.*, 2016). In this light, the microbial community residing into the amphibians' skin mucus can possibly act as a primary barrier against environmental agents; for example, there is some evidence that microbial produced compounds can act to protect their hosts from pathogens like the chytrid fungus (Becker *et al.*, 2009, 2011; Bletz *et al.*, 2013; Harris *et al.*, 2009; Pask, 2012). Therefore, it is important to investigate the diversity of this skin microbiome to understand if it can be impacted by pollution. If this is the case, amphibians living in polluted environments could come to lose a key component of their tolerance to environmental perturbations.

Metal contamination and mechanisms of toxicity

Metal contamination is recognized as having severe impacts on biodiversity all over the world. It can originate from many sources, namely industrial processes, water acidification (inducing metal ions leeching) or mining activities (Knasmüller *et al.*, 1998; Linder and Grillitsch, 2000; Nadal *et al*., 2004; Roark and Brown, 1996; Santoro *et al.*, 2008; Young and Harvey, 1991). The latter has a great impact on the soil surrounding the mine, in the water originating from it and in the surrounding water bodies contaminated by runoff and lixiviation (Knasmüller *et al.*, 1998; Macnair, 1997; Nordstrom, 2011; Shaw, 1999). Metal -enriched waters, coming from mining activities, are characterized by a diverse composition and assortment of ions, which depends on the minerals present in the bedrock of the mine (Nordstrom and Alpers, 1999; US-EPA, 1989). These waters are acidic; their pH being often 5 or lower (Bowell and Bruce, 1995; Nordstrom, 2011; Nordstrom *et al.*, 2000; Pereira *et al.* 2004; Sobral *et al.* 2013). Their effects on the surrounding environment also depend upon the degree of dilution the acid mine drainage suffers, due to rainfall or nearby rivers, exerting more severe effects near the extraction site (Nordstrom, 2011; Nordstrom and Alpers, 1999).

When interacting with living beings, metal ions usually bind to specific receptors in cell's membranes (Di Toro *et al.*, 2001; Wright, 1995). Most of the time, this process occur at the surface of respiratory organs in animals or in plant roots (but even leaves). Here, the binding ions can be pumped into the cells by active mechanisms or simply passing through by osmosis (Linder and Grillitsch, 2000; Di Toro *et al.*, 2001). In microorganisms, the mechanism of tolerance to metal can involve: (1) hydrogen sulfide production to bind metal ions resulting in insoluble sulphates; (2) chelation of metal ions by active binding to organic substances; (3) sequestration of metal ions by binding to cell surfaces or by intracellular uptake sequestration; (4) change in valence and/or active conversion into organometallic compounds; (Branco *et al*., 2008a; Branco *et al.*, 2008b; Francisco *et al*., 2002; Gadd and Griffiths, 1977; Santo *et al*., 2010; Sousa *et al*., 2013). Organisms purposely take in metal ions that are necessary for their physiological processes, like copper, zinc, iron, and other, which are essential for life (Di Toro *et al.*, 2001; Rainbow, 2007). But even essential ions will become toxic when present at high concentrations, surpassing predetermined (and species specific) physiological thresholds (Rainbow, 2007; US-EPA, 1989; Wright, 1995). Their toxicity can be exerted when the ions bind to cellular receptors, which were meant to bind with other ions with equal charge, molecular weight or ionic valence (Linder and Grillitsch, 2000). In that case, especially when the concentration of the interfering ions fairly exceeds those of the proper one, the receptors became almost fully unavailable for the latter. Mutagenic metal ions can also bind to DNA altering its functions. Both mechanisms inhibit whichever correlated physiological reaction (Goyer and Clarkson, 2001; Linder and Grillitsch,

2000). Those mechanisms can block or lessen the production of particular compounds, enzymes or others proteins needed in the target organism, possibly leading to severe consequences (Goyer and Clarkson, 2001). If lethal concentrations are not reached the target organisms could experience a variety of sublethal effects: growth inhibition and delay, malformations, metabolic deficiencies, hormonal disequilibrium, sterility and unnatural behavior (Goyer and Clarkson, 2001; Linder and Grillitsch, 2000). The degree and kind of effect depends on many different variables, as those inherent to metal speciation and those concerning the organism on which it acts (Goyer e Clarkson, 2001; Linder and Grillitsch, 2000). Indeed, affected organisms can have a lot of different strategies to better cope with metal contamination. A simple choice, for animals able to do so, is to flee the polluted areas, being spatial avoidance one of the primary responses (Lopes *et al.* 2004; Lukkari and Haimi, 2005). Indeed, an option that plants and sessile or slow moving animals cannot afford, even if plants can make their roots grow toward deeper unpolluted soil (Dickinson *et al.* 1991). When spatial escaping is not possible, populations can adapt, with physiological plastic responses (Adlassnig *et al.*, 2013; Peña-Castro *et al.*, 2004; Via *et al.* 1995), trans-generational effects (Muyssen and Janssen, 2001; Pölkki *et al.* 2012) and/or genetic inheritable factors (Dieckmann and Doebeli, 2004; *Lopes et al.* 2006; Shaw, 1999). Either way, those mechanisms often involve metabolic pathways able to discriminate toxic ions or excrete them, when they are at a higher than desired concentration (Marsden and Rainbow, 2004; Rainbow, 2002, 2007). Such kind of responses came in a variety of ways; for example, plants can expel metal ions through their roots while crustaceans can accumulate them as tiny crystals in their carapaces, ready to be disposed with the subsequent mould (Marsden and Rainbow, 2004; Rainbow and Scott, 1979). Bacteria can either accumulate metal ions in their cells or at the membrane's surface or bind them to other organic or inorganic compounds to make them not bioavailable (Gadd and Griffiths, 1979). Amphibians mainly enter in contact with metal pollutants in the aquatic medium; in which they live during embryonic and larval stages. Even when they are adult, most amphibians inhabit humid habitats, thus a constant layer of moisture surround their skin, even when they are not in the water. As a result, the diffusion of metal ions in the water is a key process to exert the toxicity of these compounds (Todd *et al.*, 2011). The jelly coat surrounding the eggs is the first barrier that ions have to surpass to get to the embryo, while in later stages, after the development of the gills, these will likely be the target organ. As adults, the skin of the amphibians is the primary barrier against external agents, although it is highly permeable and unlikely to prevent the entrance of metal ions. The barrier effect of the skin is primarily exerted by its mucus and the bacterial community living in it. The complexity of this scenario is very high; the degree of the toxic effects on amphibians is not only related to their life stage, neither only with the kind and concentration of the metal contaminating the ponds in which they live. The characteristics of the

water body impact greatly this outcome, as pH, hardness and dissolved organic matter can interact with metal ions and alter their speciation in a variety of complex ways (Adlassnig *et al.*, 2013; Freda, 1986; Freda 1991; Horne and Dunson, 1995; Rodríguez *et al.*, 2009). Nevertheless, there are examples in which the toxicity caused by metals is mainly due to the content of metal ions present in the sediment; specifically when tadpoles feed near the bottom of a contaminated pond, they can uptake a quantity of metal by ingestion and end up showing toxic effects and/or bioaccumulation (Karasov *et al.* 2009; Loumbourdis *et al.*, 2007; Sparling, 1996). The effect of metal contamination on amphibians range from death to malformations, to physiological reproductive or metamorphosis impairment, depending on the intensity of the contamination and its duration in time also. For example, chronic exposure can result in upregulation of some genes (Marques *et al.*, 2013). The most likely effects, at low metal concentrations, are the ones lowering the individuals' fitness; like reduced growth and fertility (Adlassnig *et al.*, 2013; Todd *et al.*, 2011; Zocche *et al.*, 2014); these effects can be related to mechanisms of oxidative stress too (Borković-Mitić *et al.*, 2016; Marques *et al.*, 2011).

Objectives

Within the described context, the main objective of this work was to understand the tolerance to metals of amphibian aquatic stages and of their skin associated microbiomes. To attain this major goal the following specific objectives were delineated:

a) Resume a coherent and structured understanding of the genetic erosion mechanisms, focusing on amphibians as a model;
b) Contribute to the understanding of the heritable tolerance to metal contamination in amphibians;
c) Investigate if amphibians' populations historically exposed to metals exhibit higher tolerance to this kind of contamination;
d) Assess if different environmental conditions (metal contamination) influence the bacterial diversity in amphibian´s skin.
e) Determine if bacteria strains, collected on frogs from a metal contaminated site, are more tolerant to such contamination than those from reference populations.

Acid mine drainage (AMD) was chosen as the pollutant factor and amphibians as the target group, being Perez's frog (*Pelophylax perezi*) the model species.

The study sites

Mining was an important activity in the Iberian Peninsula since the Roman Empire ages, when the Romans already extracted various metals. Among those, precious metals like silver or gold, and others like iron, copper, lead, and tin (Edmondson, 1989). The geological conformation of the region gave origin to the Iberian pyrite belt in the Devonian period (Paleozoic era, 419,2 Mya – 358,9 Mya), due to massive volcanic activity, leading to huge sulfide deposits. It is a vast mining region, about 250 km long and 30 to 50 km wide. It lays approximately from Alcácer do Sal, in Portugal, to Sevilla, in Spain, running northwest to southeast (Gibbons and Teresa, 2002). This area saw his ancient mine exploitation diminishing during the middle ages but, returning at the economy's leading activities with the industrial revolution. When industrial companies from northern Europe came to this region, they made the extraction of pyrite the pillar of this region for a century and a half. More than 250 extraction points were active in the pyrite belt at that time. The extraction continued in many areas until the end of the 19th century, declining since then (Gibbons and Teresa, 2002). Regarding the Portuguese portion of this region, the most important mines were in Aljustrel and São Domingos, both located in the Alentejo region, at both mines, iron was the main extracted metal. While the Aljustrel mine is still active, São Domingos was abandoned for more than 50 years, but still highly impacts the surroundings (Pereira *et al.* 2004; Sobral *et al.* 2013). Nevertheless, even if the impact of mining activities is more evident at São Domingos, its effect is present also in the Aljustrel district (Fig.1.1 and 1.2). More than a century of mining exposed sulfur rich minerals and led to huge deposits of slag accumulating around the mine pits. Over the years, the weather acted on those rocks and the rain pouring through it was enriched with metal and hydrogen ions. At present, these waters, coming from the mining spots, constitute acid drainage (AMD) that reaches pH values near 2 (Costa *et al.*, 2016; Pereira *et al.* 2004; Sobral *et al.* 2013). The drainage from the Aljustrel mine results in a yellow/red colored water river (Ribeira de Água Forte, Fig. 1.1), while in São Domingos the same sort of water flows through a 10 km valley (Fig. 1.2). The Spanish portion of the Iberian pyrite belt was equally exploited, for example, in the mines of the Alcudia valley, Almaden and Horcajo; all located in the Province of Ciudad Real (Castilla-La Macha region) (Fig. 1.3). This mining area was the major lead producer in Spain during the second half of the 19th century and the Alcudia Valley was one of the most important districts (Rodríguez *et al.*, 2009). The soils analyzed at the Almadén mine, now converted into a museum since 2000, showed increasing mercury levels, with a gradient in direction to the mine, caused by the erosion of the mineral deposits; mercury extracted from Almadén mine produced the largest amount of liquid mercury ever processed in our planet (Higueras *et al.*, 2006; Lindberg *et al.*, 1978). The Horcajo lead mine had a history similar to that of the Alcudia

valley (Baranda, 1994). A study reported analysis of high lead contamination in the soils, which was transferred into plants growing on it (for example grass of the *Gramineae* family or trees of the genus *Quercus*) and animals feeding on them (*Cervus elaphus*) (Reglero *et al.*, 2008). In this context, six metal polluted locations, and seven unpolluted locations were chosen as sampling sites for our studies about Perez's frog (eggs, and skin microbiome in Portugal; tadpoles in Spain) tolerance to AMD.

Fig.1.1: Água Forte river, impacted by AMD coming from the Aljustrel mining site.

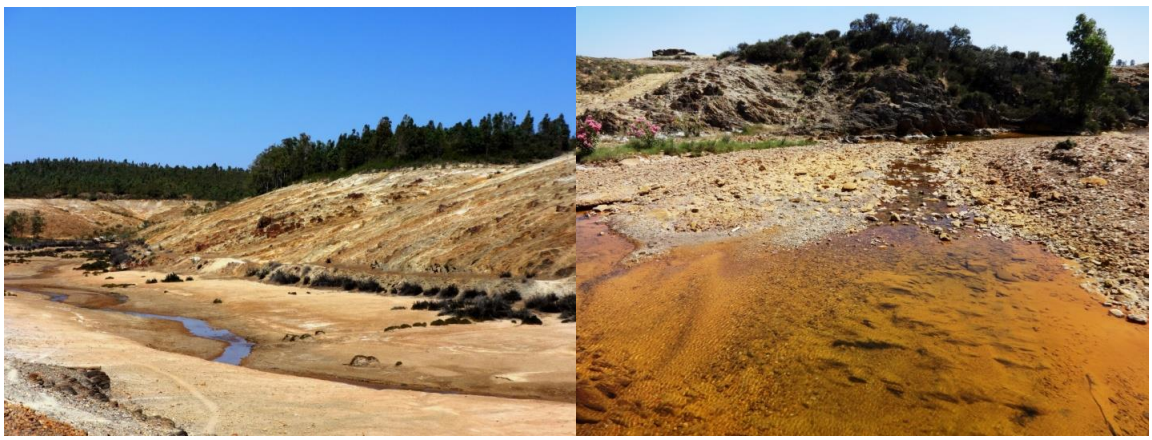


Fig.1.2: The effluent running through São Domingos mining site.

Fig. 1.3: Minas de Almadén (left), and Arroyo de Valdefuentes in the Alcudia area (right).

The model organism

To perform the present work, the Perez's frog (*Pelophylax perezi*, López-Seoane, 1885) is an advantageous model species, because it is easily encountered in the field and its larval stages can be maintained in the laboratory with no great effort. The Perez's frog may inhabit eutrophic or even polluted habitats (Marques *et al.*, 2013; Sillero and Ribeiro, 2010). Its ability to bear environmental stressors drove our choice of this species, for it is ideal to compare responses between pristine and polluted sites. Furthermore, there is no or little information about the microbiome composition of this species of frog. This frog is endemic and diffused in the Iberian Peninsula (Portugal and Spain) and southern France, though its northern range limit is still open to questions. The species was introduced in the Madeira, Balearic, Canary, and Azores islands and in the United Kingdom (Arnold and Ovenden, 2002; Loureiro *et al.*, 2008). Its conservation status is of no concern and, more importantly, it can inhabit even highly polluted areas (Arnold and Ovenden, 2002; Bosch *et al.*, 2009; Sillero and Ribeiro, 2010). The species was first described and attributed at the genus *Rana*, only subsequently being relocated to the newborn genus *Pelophilax*, which comprise all others former "*Rana* species", like *Pelophylax lessonae*, *Pelophylax ridibundus* and *Pelophylax kl. esculentus*. The Perez's frog *Pelophylax perezi* is a rather slender frog, agile and fast swimmer. It can measure up 10 cm snout to vent, but it is usually smaller and grows as large only after 2-3 years of life, also the males are 1-2 cm (snout to vent) smaller than females. This frog is usually green, with various darker spots on the dorsal side, but its color variability is huge and it is possible to encounter light green individuals as well as dark brown ones. A distinctive feature is the presence of 3 dorsal lines. The central line runs between the eyes to the rear in the middle of the dorsal region, while the others two go from behind the eye to the attachment of the hind-legs. The former is always present and better marked, while the remaining are often not quite marked or even absent. The lines use to be lighter than the dominant color of the frog but, once again, their color variability is very high (Arnold and Ovenden, 2002) (Fig. 1.4). The Perez's frog hibernates during autumn and winter, in the riverbanks or lakes shores. It becomes active at early spring, as soon as the days lengthen, and males start calling for mates soon after. Their reproductive season typically spans from March to June in the Iberian Peninsula but can be longer or shorter at other latitudes (Arnold and Ovenden, 2002). The spawns typically present 50 to 200 eggs, grouped together, enveloped in gelatinous coats and secured under aquatic plants leaves (Arnold and Ovenden, 2002; Bosch *et al.*, 2009). The eggs develop into swimming larvae in about a week, in the field (Arnold and Ovenden, 2002; Bosch *et al.*, 2009). The tadpoles develop into herbivore grazers and occasional omnivores, starting their metamorphosis about two months after emerging from their egg (Arnold and Ovenden, 2002; Bosch *et al.*, 2009). *Pelophylax perezi* thrives

in stream, rivers, lakes, ponds, and agricultural ditches, usually selecting slow current zones. Its temperatures limits go up to 35°C and down to 3°C and this frog can live at altitudes up to 2300 meters, in Sierra Nevada (Spain) (Arnold and Ovenden, 2002; Loureiro *et al.*, 2008).

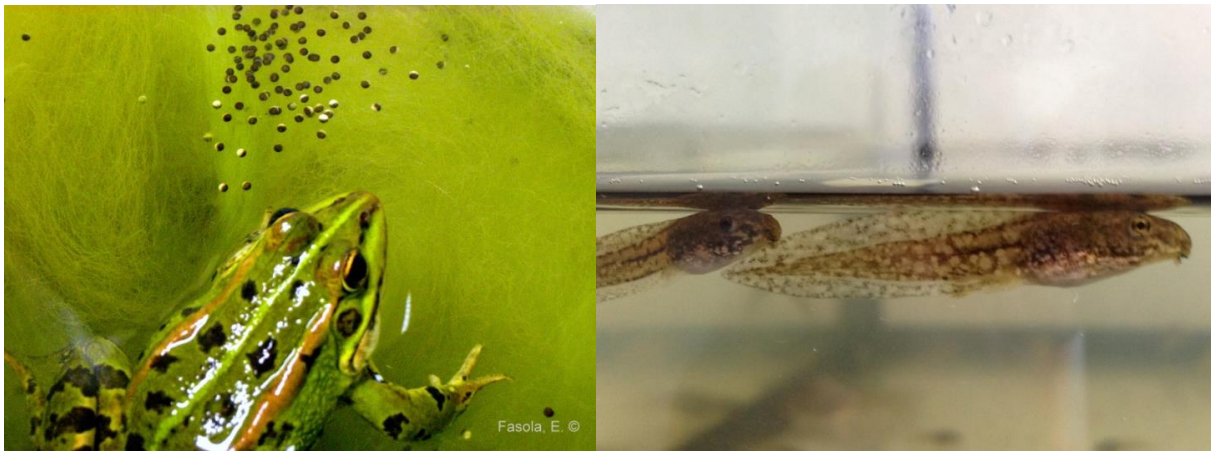


Fig.1.4: A Perez's frog adult with its eggs (left) and tadpoles (Gosner stage 27) in the laboratory (right).

Detailed description of each chapter

Chapter 1 – Corresponds to the general introduction of this document and provides an overall view of the basic knowledge underlying the purpose of the research performed during this work, as well as the explanation of its intended objectives.

Chapter 2 – This chapter tackles the first specific objective (a). It joins the previous contributions about genetic erosion theory and looked at them both with a broad view, open to various applications, and with a special focus on amphibians. It is pointed out that genetic erosion is the loss of populations' genetic variation: the loss of genotypes determining a specific trait or set of traits. It is reported, in a coherent way, studies focused on genetic variability altered by microevolutionary processes. As much as a third of known amphibian species is reported as endangered. A decreasing genetic variability can lower their future adaptability, ultimately leading to local extinction. This chapter highlights how amphibians' populations are naturally fragmented, often highly inbred, or bottlenecked; and how those populations already harbor lowered genetic diversity (in the light of the studies available in literature). Amphibians populations could so be a model to assess how contaminant-driven effects can decrease their genetic variability. This chapter specify that contaminant-driven genetic erosion impacts: 1) fitness, 2) environmental plasticity, 3) co-tolerance mechanisms, 4) trade-off mechanisms, and 5) tolerance to pathogens; in amphibian populations.

Chapter 3 - This third chapter was aimed to understand how inheritance, of genetically determined, tolerance to metal contamination stands behind individuals' sensitivity. Each individual expression, of tolerance to metal, would be due to the specific conditions of the trait's dominance status. This chapter investigated the recessive tolerance inheritance (working-) hypothesis, which is verified if tolerance is due to the action of a recessive or incompletely dominant trait. In this case, contaminant-driven genetic erosion could be able to completely remove a large portion of the genetic variability, in a population impacted by metal contamination. The results seem to support this hypothesis, as we found the genetically determined tolerance to AMD, tested in *Pelophylax perezi* eggs, to be determined by incomplete dominance mechanism.

Chapter 4 - The fourth section is a further investigation of the mechanisms of genetically determined tolerance. Carried out with the same methodologies but in respect of the toxicity of a single metal ion: copper. Indeed, copper is one of the main components of AMD coming from Aljustrel and São Domingos mines. Focusing on a single metal is also useful to exclude the confounding factors deriving from the study of a highly complex mixture, with such a low pH, like AMD is. Using a copper solution, it is possible to perform the toxicity assays characterized only by an excess of copper ions. The pH of such solution, being almost neutral (near pH 7) thus excluding the potential effect of a low pH. To further corroborate the observed patterns this work was repeated, with the same procedures and in the same frogs' population, in two different years: 2014 and 2016. The genetically determined tolerance to copper, was found again to be likely determined by incomplete dominance. This chapter also includes a short review about the various tolerance mechanisms to metals. Being able to insert our own findings into a broader context.

Chapter 5 - The fifth chapter studies the tolerance to metal contamination in historically-exposed populations of Perez's frog tadpoles. These were sampled in sites known to be impacted by AMD coming from Minas de Alcudia, Minas de Almadén and Minas de Horcajo (Castilla-La-Mancha region, south-western Spain). This work intended to explore if, exposure to low levels of mercury and lead through the aquatic developmental stages could enable the tadpoles to better tolerate a subsequent stronger (but short lasting) metal contamination pulse. No evidence on that was found. Tadpoles, already living in contact with low Hg and Pb levels, did not cope better than the controls to controls to an exposure to high concentrations of these two metals.

Chapter 6 - This chapter investigated the diversity of *Pelophylax perezi* skin microbiome. Researchers increasingly understand that the microbial community, being hosted by a larger

organism, is an integrating factor in the host's physiology and immune system's responses. Some bacterial strains have been found to provide their hosts with substances able to inhibit infectious agents or pathogens. For example, violacein, found in north American salamanders' skin microbiome, can lower the growth of *Bathracochytrium dendrobatidis* (Becker *et al.*, 2009). In this context, some studies followed, characterizing skin microbial communities for various amphibian species, sampled in natural parks or pristine sites. The research group of the university of Aveiro started to publish in 2016, to address if such microbial communities could be impacted or altered (as much as their hosts) by pollution. Indeed, this study confirms and extends the previous findings. The obtained results suggest that acid mine drainage can alter the composition of the bacteria community in amphibians' skin, both lowering their diversity and shifting the composition of such a community; the results being corroborated both by cultivable approach and microbiome next generation sequencing (NGS; 16S rRNA gene-based metagenomics). This is not only a loss of biodiversity related to bacterial species, but also a loss of a potentially precious physiological symbiosis relationship. Which in turn is part of the ecological diversity of the Perez's frog populations. This study reported, for the studied populations and for the first time, different microbial communities' compositions between male and female individuals. Thus, it is important to consider both unpolluted and contaminated sites, with matching sex-ratio, samples to deepen our understanding of the above-mentioned dynamics. Furthermore, the growth inhibition assay, conducted on isolated bacterial strains exposed to AMD, showed that there is no difference in the level of tolerance to AMD of the strains isolated from reference or metal polluted sites. However, a 75% AMD concentration succeeded in inhibit the growth of all the isolated strains (except for four genera: *Erwinia, Serratia, Stenotrophomonas* and *Phyllobacterium*).

Chapter 7 – This chapter corresponds to the general conclusion that resumes all the evidences obtained during this work and connects them into a broader framework.

References

ABLER, David Gerrard; SHORTLE, J. S. - Environmental policies for agricultural pollution control. [S.l.] : **CABI Publishing** (2001). ISBN 00851993990.

ADLASSNIG, Wolfram; SASSMAN, Stefan; GRAWUNDER, Anja; PUSCHENREITER, Markus; HORVATH, Amadea; KOLLER-PEROUTKA, Marianne - Amphibians in metal-contaminated habitats. **Salamandra**. 49:3 (2013) 149–158.

Agenda 21 : programme of action for sustainable development ; Rio Declaration on Environment and Development ; Statement of Forest Principles: The final text of agreements negotiated by governments at the United Nations conference

on environment and devel - Em . Rio de Janeiro, Brazil. : New York, NY: United Nations Dept. of Public Information (1993).

ALFORD, Ross A.; RICHARDS, Stephen J. - Global amphibian declines: a problem in applied ecology. **Annual Review of Ecology and Systematics**. 30:1999 (2007) 133–165.

ARNOLD, Nicolas; OVENDEN, Denys - Reptiles and Amphibians of Britain and Europe. [S.l.] : Collins; New edition (2002).

BALÁŽ, Vojtech; VOROS, Judit; IVIS, Petr; VOJAR, Jiri; HETTYEY, Attila; SOS, Endre; DANKOVICS, Róbert; JEHLE, Robert; CHRISTIANSEN, Ditte G; CLARE, Frances; FISHER, Matthew C; GARNER, Trenton W J; BIELBY, Jon. - Assessing Risk and Guidance on Monitoring of *Batrachochytrium dendrobatidis* in Europe through Identification of Taxonomic Selectivity of Infection. **Conservation biology : the journal of the Society for Conservation Biology**. ISSN 1523-1739. (2013) 1–11. doi: 10.1111/cobi.12128.

BARANDA, Borja Sainz - The Horcajo mines. **Mineralogical Record**. 25:1 (1994) 1–21.

BAUD, D. R.; BECK, M. L. - Interactive effects of UV-B and copper on spring peeper tadpoles (Pseudacris crucifer). **Southeastern Naturalist**. 4 (2005) 15–22.

BECKER, Matthew H.; BRUCKER, Robert M; CHRSTIAN, R; HARRIS, Reid N; MIMBIOLE, Kevin P C; SCHWANTES, Christian R - The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus. **Applied and Environmental Microbiology**. 75:21 (2009) 6635–6638. doi: 10.1128/AEM.01294-09.

BECKER, Matthew; HARRIS, Reid N; MINBIOLE, Kevin P C; GRATWICKE, Brian - Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. **EcoHealth**. ISSN 16129210. 8:4 (2011) 501–506. doi: 10.1007/s10393-012-0743-0.

BEEBEE, T. J. C. - Conservation genetics of amphibians. **Heredity**. . ISSN 0018-067X. 95:6 (2005) 423–7. doi: 10.1038/sj.hdy.6800736.

BEEBEE, Trevor J. C.; GRIFFITHS, Richard A. - The amphibian decline crisis: A watershed for conservation biology? **Biological Conservation**. . ISSN 00063207. 125:3 (2005) 271–285. doi: 10.1016/j.biocon.2005.04.009.

BIELBY, J.; BOVERO, S.; AGELINI, C.; FAVELLI, M.; GAZZANIGA, E.; PERKINS, M.; SOTGIU, G.; TESSA, G.; GARNER, T. W. J. - Geographic and taxonomic variation in Batrachochytrium dendrobatidis infection and transmission within a highly endemic amphibian community. **Diversity and Distributions**. ISSN 13669516. 19:9 (2013) 1153–1163. doi: 10.1111/ddi.12085.

BLAUSTEIN, AR; BANCROFT, BA - Amphibian population declines: evolutionary considerations. **BioScience**. 57:5 (2007).

BLAUSTEIN, AR; WAKE, DB; SOUSA, WP - Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. **Conservation Biology** (1994).

BLETZ, Molly C.; LOUNDON, Andrew H.; HARRIS, Reid N. - Mitigating amphibian chytridiomycosis with bioaugmentation: Characteristics of effective probiotics and strategies for their selection and use. **Ecology Letters**. ISSN 1461023X. 16:6 (2013) 807–820. doi: 10.1111/ele.12099.

BOSCH, J.; TEJEDO, M.; BEJA, P.; MARTINEZ-SOLANO, I.; SALVADOR, A.; GARCIA-PARIS, M.; RECUERO Gil, E.; BEEBEE, T.J.C. - *Pelophylax perezi*. **The IUCN Red List of Threatened Species.** [On line], available at: http://dx.doi.org/10.2305/IUCN.UK.2009.RLTS.T58692A11812894.en.

BOWELL, R. J.; BRUCE, I. - Geochemistry of iron ochres and mine waters from Levant Mine, Cornwall. **Applied**

Geochemistry. ISSN 08832927. 10:2 (1995) 237–250. doi: 10.1016/0883-2927(94)00036-6.

BOYCE, M. S. - Population viability analysis. **Ann. Rev. Ecol Syst.** 23 (1992) 481–506.

BORKOVIĆ-MITIĆ, Slavica S.; PROKIC, M.; SAICIC, Zorica S. - Biomarkers of oxidative stress and metal accumulation in marsh frog (*Pelophylax ridibundus*). **Environmental Science and Pollution Research**. ISSN 16147499. 23:10 (2016) 9649–9659. doi: 10.1007/s11356-016-6194-3.

BRANCO, Rita; CHUNG, Ana Paula; VASCONCELO MORAIS, Paula; ZHITCOVIC, Anatoly - The chromate-inducible chrBACF operon from the transposable element TnOtChr confers resistance to chromium(VI) and superoxide. **Journal of bacteriology**. ISSN 1098-5530. 190:21 (2008) 6996–7003. doi: 10.1128/JB.00289-08.

BRANCO, Rita; CHUNG, Ana-Paula; VASCONCELO MORAIS, Paula V - Sequencing and expression of two arsenic resistance operons with different functions in the highly arsenic-resistant strain Ochrobactrum tritici SCII24T. **BMC microbiology** . ISSN 1471-2180. 8:1 (2008) 95. doi: 10.1186/1471-2180-8-95.

BRIDGES, C. M. - Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). **Archives of Environmental Contamination and Toxicology**. 39 (2000) 91–96.

BRÜHL, C; SCHMIDT, T; PIEPER, S; ALSCHER, A - Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? **Scientific reports** (2013) 1–4. doi: 10.1038/srep01135.

CARR, L. W.; FAHRIG, L. - Impact of road traffic on two amphibian species of differing vagility. **Conservation Biology**. 15 (2001) 1071–1078.

CBD - **Convention on Biological Diversity** [On line], available at: http://www.cbd.int/.

CHANNELL, R.; LOMOLINO, M. V. - Dynamic biogeography and conservation of endangered species. **Nature**. 403 (2000) 84–86.

CHEN, Shao-Yu; ZHANG, Yi-Jun; WANG, Xiu-Ling; QU, Liang-Hu; ZHOU, Hui; Sun, JIAN-YUN; Xue, YAN; Zhang, Peng - Extremely Low Genetic Diversity Indicating the Endangered Status of *Ranodon sibiricus* (Amphibia: Caudata) and Implications for Phylogeography. **PLoS ONE**. ISSN 1932-6203. 7:3 (2012) e33378. doi: 10.1371/journal.pone.0033378.

CHOI, Seong Yeol; YOON, Kyoung Hye; LEE, Jin Il; MITCHELL, Robert J. - Violacein: Properties and production of a versatile bacterial pigment. **BioMed Research International**. ISSN 23146141. (2015). doi: 10.1155/2015/465056.

COLLINS, James P.; STORFER, Andrew - Global amphibian declines: sorting the hypotheses. **Diversity and Distributions**. 9 (2003) 89–98.

CORN, P. S.; VERTUCCI, F. A. - Descriptive risk assessment of the effects of acidic deposition on Rocky Mountain amphibians. **Journal of Erpethology**. 26 (1992) 361–369.

COSTA, S.; NEVES PROENÇA, D.; RIBEIRO, R; LOPES, I; VASCONCELO MORAIS, P - Diversity of cutaneous bacterial community of Pelophylax perezi populations inhabiting different environments. **Science of the Total Environment**. ISSN 1098-6596. in press (2016). doi: 10.1016/j.scitotenv.2016.07.230.

COUTELLEC, Marie-Agnès; BARATA, Carlos - An introduction to evolutionary processes in ecotoxicology. **Ecotoxicology (London, England)**. ISSN 1573-3017. 20:3 (2011) 493–6. doi: 10.1007/s10646-011-0637-x.

CUSHMAN, Samuel A. - Effects of habitat loss and fragmentation on amphibians: A review and prospectus. **Biological Conservation**. ISSN 00063207. 128:2 (2006) 231–240. doi: 10.1016/j.biocon.2005.09.031.

DÍAZ-RAVIÑA, M.; BÅÅTH, E.; FROSTEGÅRD, A. - Multiple heavy metal tolerance of soil bacterial communities and its measurement by a thymidine incorporation technique. **Applied and environmental microbiology**. ISSN 0099-2240. 60:7 (1994) 2238–47.

DICKINSON, N. M.; TURNER, A. P.; LEPP, W. N. - How do trees and other long-lived plants survive in polluted

environments? **Functional Ecology**. 5:1 (1991) 5–11. doi: 10.2307/2389550.

DIECKMANN, U.; DOEBELI, M. - Adaptive dynamics of speciation: sexual populations. **Adaptive speciation** (2004).

DIETZ, J. M.; DIETZ, L. A.; NAGAGATA, E. Y. - The effective use of flagship species for conservation of biodiversity: the example of lion tamarins in Brazil. Em **Creative conservation: Interactive management of wild and captive animals.** London : Chapman&Hall (1994) p. 32–49.

DI TORO, M; ALLEN, H E; BERGMAN, H L; MEYER, J S; PAQUIN, P R; SANTORE, R C - Biotic ligand model of the acute toxicity of metals. 1. Technical basis. **Environmental toxicology and chemistry / SETAC**. ISSN 07307268. 20:10 (2001) 2383–2396. doi: 10.1897/1551-5028(2001)020<2383:blmota>2.0.co;2.

DOBSON, Alleson; COTTER, Paul D.; ROSS, R.; HILL, Colin - Bacteriocin production: A probiotic trait? **Applied and Environmental Microbiology**. ISSN 00992240. 78:1 (2012) 1–6. doi: 10.1128/AEM.05576-11.

EDMONDSON, J. C. - Mining in the later Roman Empire. **The Journal of Roman Studies**. 79 (1989) 84–102.

ENTWISTLE, A.; BOWEN-JONES, E. - Identifying appropriate flagship species: The importance of culture and local contexts. **Oryx**. 36:2 (2002) 189–195.

FAHRIG, L. - Relative effects of habitat loss and fragmentation on population extinction. **The Journal of Wildlife Management**. 61 (1997) 603–610.

FASOLA, E.; RIBEIRO, R.; LOPES, I. - Microevolution due to pollution in amphibians: A review on the genetic erosion hypothesis. **Environmental Pollution**. . ISSN 18736424 02697491. 204 (2015). doi: 10.1016/j.envpol.2015.04.027.

FEDORENKOVA, A.; VONK, J. - Ranking ecological risks of multiple chemical stressors on amphibians. **Environmental Toxicology and Chemistry**. 31:6 (2012) 1416–1421. doi: 10.1002/etc.1831.

FERNÁNDEZ-BENÉITEZ, María José; ORTIZ-SANTALIETRA, Manuel Eloy; LIZANA, Miguel; DIEGUEZ-URIBEONDO, Javier - Differences in susceptibility to *Saprolegnia* infections among embryonic stages of two anuran species. **Oecologia**. . ISSN 1432-1939. 165:3 (2011) 819–26. doi: 10.1007/s00442-010-1889-5.

FISCHER, Anke; YOUNG, Juliette C. - Understanding mental constructs of biodiversity: Implications for biodiversity management and conservation. **Biological Conservation**. ISSN 00063207. 136:2 (2007) 271–282. doi: 10.1016/j.biocon.2006.11.024.

FISKER, Karina V.; SORENSEN, Jesper G; DAMGAARD, Christian; PEDERSEN, Knud Ladegaard; HOLMSTRUP, Martin - Genetic adaptation of earthworms to copper pollution: is adaptation associated with fitness costs in *Dendrobaena octaedra*? **Ecotoxicology (London, England)**. ISSN 1573-3017. 20:3 (2011) 563–573. doi: 10.1007/s10646-011-0610-8.

FLEISHMAN, Erica; NOSS, Reed F.; NOON, Barry R. - Utility and limitations of species richness metrics for conservation planning. **Ecological Indicators**. . ISSN 1470160X. 6:3 (2006) 543–553. doi: 10.1016/j.ecolind.2005.07.005.

FOLT, C. L.; CHEN, C.Y.; MOORE, M.V.; BURNAFORD, J. - Synergism and antagonism among multiple stressors. **Limnology and Oceanography**. 4 (1999) 864–877.

FRANCISCO, R.; ALPOIM, M. C.; MORAIS, P. V. - Diversity of chromium-resistant and -reducing bacteria in a chromium-contaminated activated sludge**. Journal of Applied Microbiology**. ISSN 13645072. 92:5 (2002) 837–843. doi: 10.1046/j.1365-2672.2002.01591.x.

FREDA, J. - The influence of acidic pond water on amphibians: A review. **Water Air Soil Pollution.** 20 (1986) 439–450.

FREDA, J. - The effects of aluminum and other metals on amphibians. **Environmental Pollution.** 71 (1991) 305–328.

GADD, Geoffrey Michael; GRIFFITHS, Alan J. - Microorganisms and Heavy Metal Toxicity. **Microbial ecology**. ISSN 0095-3628. 4:4 (1977) 303–317. doi: 10.1007/BF02013274.

GHALAMBOR, C. K.; McKAY, J. K.; CARROL, S. P.; REZNICK, D. N. - Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. **Functional Ecology**. ISSN 0269-8463. 21:3 (2007) 394–407. doi: 10.1111/j.1365-2435.2007.01283.x.

GIBBONS, Wes; TERESA, Moreno - Economic and environmental geology. In **The geology of Spain.** ISBN 978-1-86239-127-7. (2002) p. 478–484.

GOMEZ-MESTRE, I.; TEJEDO, M.; RAMAYO, E.; ESTEPA, J. - Developmental alterations and osmoregulatory physiology of a larval anuran under osmotic stress. **Physiol. Biochem. Zool.** 77 (2004) 267–274.

GOYER, R. A.; CLARKSON, T. W. - Toxic effects of metals. Em KLAASSEN, C. D. (Ed.) - **Casarett and Doull's toxicology: the basic science of poisons.** [S.l.] : McGraw-Hill Professional, New York, NY, USA., 2001. p. 811–867.

HALL, R. J.; HENRY, P. F. P. - Assessing effects of pesticides on amphibians and reptiles: status and needs. **Herpetological Journal**. 2 (1992) 65–71.

HARRIS, RN; BRUCKER, RM; WALKE, JB - Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. **THE ISME JOURNAL**. . ISSN 1751-7362. 3:7 (2009) 818–824. doi: 10.1038/ismej.2009.27.

HATCH, A. C.; BLAUSTEIN, A. R. - Combined effects of UV-B radiation and nitrate fertilizer on larval amphibians. **Ecological Applications**. 13 (2003) 1083–1093.

HEDRICK, Philip W.; KALINOWSKI, Steven T. – Inbrreding depression in conservation biology. **Annual Review of Ecology and Systematics**. 31 (2000) 139–162.

HIGUERAS, P.; OYARZUN, R.; LILLO, J.; LORENZO, S. - The Almadén district (Spain): anatomy of one of the world's largest Hg-contaminated sites. **Science of the Total Environment**. ISSN 00489697. 356:1–3 (2006) 112–124. doi: 10.1016/j.scitotenv.2005.04.042.

HORNE, M. T.; DUNSON, W. A - The interactive effects of low pH, toxic metals, and DOC on a simulated temporary pond community. **Environmental Pollution**. ISSN 0269-7491. 89:2 (1995) 155–61.

HOULAHAN, J. E.; FINDLAY, C.S.; SCHMIDT, B. R.; MEYER, A. H.; KUZMIN, S. L. - Quantitative evidence for global amphibian population declines. **Nature**. 404 (2000) 752–755.

HUEY, D. W.; BEITINGER, T. L. - Toxicity of nitrite of the Salamander *Ambystoma texanum*. **Bull. Environ. Contam. Toxicol.** 25 (1980) 909–912.

HUA, Jessica; MOREHOUSE, Nathan I.; RELYEA, Rick - Pesticide tolerance in amphibians: Induced tolerance in susceptible populations, constitutive tolerance in tolerant populations. **Evolutionary Applications**. . ISSN 17524563. 6:7 (2013) 1028–1040. doi: 10.1111/eva.12083.

IRELAND, P. H. - Separate effects of acid-derived anions and cations on growth of larval salamanders of Ambystoma maculatum. **Copeia** (1991) 132–137.

ISLAM, Md Shahidul; TANAKA, Masaru - Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. **Marine Pollution Bulletin**. ISSN 0025326X. 48:7–8 (2004) 624–649. doi: 10.1016/j.marpolbul.2003.12.004.

IUCN On line, availble at: http://www.iucn.org/.

JOBLING, S.; BAYNES, A.; GARNER, T. W. J. - Frogs at risk and possible implications for humans? **CHEM Trust**. October (2013).

KARASOV, W. H.; CHEN, T.; GROSS, J. A. - Chronic exposure to pentavalent arsenic of larval leopard frogs (*Rana*

pipiens): bioaccumulation and reduced swimming performance. **Ecotoxicology.** 18:2009) 587–593.

KELLER, LF; WALLER, DM - Inbreeding effects in wild populations. **Trends in Ecology & Evolution** (2002).

KIESECKER, Joseph M.; BLAUSTEIN, Andrew R.; BELDEN, L. K. - Complex causes of amphibian population declines. **Nature**. 410 (2001) 681–684.

KNASMÜLLER, S; GOTTMAN, E; GOD, R; KUNDI, M - Detection of genotoxic effects of heavy metal contaminated soils with plant bioassays. **Mutation research**. ISSN 0027-5107. 420:1–3 (1998) 37–48. doi: 10.1016/S1383-5718(98)00145-4.

LAAN, R.; VERBOOM, B. - Effects of pool size and isolation on amphibian communities. **Conservation biology**. 54 (1990) 251–262.

LINDBERG, S. E.; JACKSON, D.R.; LUND, J. R. - Atmospheric emission and plant uptake of mercury from agricultural soils near the Almadén mercury mine. **Journal of Environmental Quality**. 8:4 (1978) 572–578. doi: 10.2134/jeq1979.00472425000800040026x.

LINDER, G.; GRILLITSCH, B. - Ecotoxicology of metals. **Ecotoxicology of Amphibians and Reptiles, SETAC** (2000).

LOPES, Isabel; MARTINS, Nelson; BAIRD, Donald J; RIBEIRO, Rui. - Genetic Erosion and population resilience in *Daphnia Longispina* O.F. Muller under simulated predation and metal pressures. **Environmental Toxicology and Chemistry**. 28:9 (2009) 1912–1919.

LOPES, Isabel; BAIRD, Donald J.; RIBEIRO, Rui - Avoidance of copper contamination by field populations of *Daphnia longispina*. **Environmental toxicology and chemistry / SETAC**. ISSN 0730-7268. 23:7 (2004) 1702–1708. doi: 10.1897/03-231.

LOPES, Isabel; BAIRD, Donald J.; RIBEIRO, Rui - Genetic adaptation to metal stress by natural populations of *Daphnia longispina*. **Ecotoxicology and environmental safety**. ISSN 0147-6513. 63:2 (2006) 275–85. doi: 10.1016/j.ecoenv.2004.12.015.

LOUMBOURDIS, N. S.; KOSTAROPOULOS, Iason; THEODOROPOLOU, Basiliki; KALMANTI, Dimitra - Heavy metal accumulation and metallothionein concentration in the frog *Rana ridibunda* after exposure to chromium or a mixture of chromium and cadmium. **Environmental pollution** (Barking, Essex). ISSN 0269-7491. 145:3 (2007) 787–792. doi: 10.1016/j.envpol.2006.05.011.

LOUREIRO, A.; ALMEIDA, N.F.; CARRETERO, M.A.; PAULO, O.S.- **Atlas de Anfíbios e Répteis de Portugal.** [S.l.] : INOVA, Portugal., 2008

LUKKARI, Tuomas; HAIMI, Jari - Avoidance of Cu- and Zn-contaminated soil by three ecologically different earthworm species. **Ecotoxicology and Environmental Safety**.. ISSN 01476513. 62:1 (2005) 35–41. doi: 10.1016/j.ecoenv.2004.11.012.

LUQUET, E. LENA, J.; DAVID, P.; JOLY, P.; LENGAGNE, T.; PERRIN, N.; PLENET, S. - Consequences of genetic erosion on fitness and phenotypic plasticity in European tree frog populations (*Hyla arborea*). **Journal of evolutionary biology**. ISSN 1420-9101. 24:1 (2011) 99–110. doi: 10.1111/j.1420-9101.2010.02138.x.

LUQUET, E; DAVID, P; LENA, J; JOLY, P; KONECNY, L; DUFRESNES, C; PERRIN, N; PLENET, S - Heterozygosity-fitness correlations among wild populations of European tree frog (*Hyla arborea*) detect fixation load. **Molecular ecology**. ISSN 1365-294X. 20:9 (2011) 1877–1887. doi: 10.1111/j.1365-294X.2011.05061.x.

LUQUET, E.; LENA, J.; DAVID, P.; PRUNIER, J.; JOLY, P.; LENGAGNE, T.; PERRINAND, N.; PLENET, S. -

Within- and among-population impact of genetic erosion on adult fitness-related traits in the European tree frog Hyla arborea. **Heredity**. 110 (2013) 347–354. doi: 10.1038/hdy.2012.110.

MACE, G.; NORRIS, K.; FITTER, A. H. - Biodiversity and ecosystem services: a multilayered relationship. **Trends in Ecology & Evolution**. 27:1 (2012) 19–26. doi: http://dx.doi.org/10.1016/j.tree.2011.08.006.

MACÌAS, G.; MARCO, A.; BLAUSTEIN, A. R. - Combined exposure to ambient UVB radiation and nitrite negatively affects survival of amphibian early life stages. **Science of the Total Environment**. 385 (2007) 55–65.

MACNAIR, M. N. - The evolution of plants in metal-contaminated environments. Em VERLAG, BIRKHAUSER (Ed.) - **Environmental Stress. Adaptation and Evolution, by Bijlsma R. and Loeschcke V.** Basel, Switzerland : [s.n.]. p. 3–24.

MARQUES, Sérgio M.; ANTUNES, Sara C; NUNES, Bruno; GONÇALVES, Fernando; PEREIRA, Ruth - Antioxidant response and metal accumulation in tissues of Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine. **Ecotoxicology** (London, England). ISSN 1573-3017. 20:6 (2011) 1315–27. doi: 10.1007/s10646-011-0688-z.

MARQUES, Sérgio M.; CHAVES, Sandra; GONÇAÇVES, Fernando; PEREIRA, Ruth - Differential gene expression in Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine. **Ecotoxicology and environmental safety**. ISSN 1090-2414. 87 (2013) 115–9. doi: 10.1016/j.ecoenv.2012.10.010.

MARSDEN, I.; RAINBOW, P. - Does the accumulation of trace metals in crustaceans affect their ecology—the amphipod example? **Journal of Experimental Marine Biology and Ecology**. ISSN 00220981. 300:1–2 (2004) 373–408. doi: 10.1016/j.jembe.2003.12.009.

MAZANTI, L.; SPARLING, D.W.; RICE, C.; BIALEK, K.; STEVENSON, C.; TEELS, B. - Synergistic effects of a combined exposure to herbicides and an insecticide in Hyla versicolor. In LINDER, G. I. - Multiple Stressor Effects in Relation to Declining Amphibian Populations. [S.l.] : (Eds.) **American Society for Testing and Materials** (2003).

MCKINNEY, M. L. - Urbanization, biodiversity, and conservation. **Bioscience**. 52:2002) 883–890.

MEDINA, MH; CORREA, JA; BARATA, Carlos - Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. **Chemosphere**. 67:2007) 2105–2114. doi: 10.1016/j.chemosphere (2006) 12.024.

MILLER, James R. - Biodiversity conservation and the extinction of experience. **Trends in Ecology and Evolution**. ISSN 01695347. 20:8 (2005) 430–434. doi: 10.1016/j.tree.2005.05.013.

MUYSSEN, B. T. A.; JANSSEN, C. R. - Multi-generation zinc acclimation and tolerance in Daphnia magna: implications for water quality guidelines and ecological risk assessment. **Environmental Toxicology and Chemistry**. 20:2001) 2053–2060.

MYERS, Norman *et al.* - Biodiversity hotspots for conservation priorities. **Nature**. . ISSN 00280836. 403:6772 (2000) 853–858. doi: 10.1038/35002501.

NADAL, M.; SCHUHMACHER, M.; DOMINGO, J. L. - Metal pollution of soils and vegetation in an area with petrochemical industry. **Science of the Total Environment**. . ISSN 00489697. 321:1–3 (2004) 59–69. doi: 10.1016/j.scitotenv.2003.08.029.

NORDSTROM, D. K. *et al.* - Negative pH and extremely acidic mine waters from Iron Mountain, California. **Environ. Sci. Technol.** 34:2 (2000). doi: 10.1021/es990646v.

NORDSTROM, D. K. - Mine Waters : Acidic to Circumneutral. **Elements**. . ISSN 1811-5209. 7:6 (2011) 393–398. doi: 10.2113/gselements.7.6.393.

NORDSTROM, D. K.; ALPERS, C. N. - Geochemistry of acid mine waters. In PLUMLEE, G. S.; LOGSDON, M. J. (Eds.) - **The Environmental Geochemistry of Mineral Deposits.** [S.l.] : Reviews in Economic Geology 6A, (1999) p.

133–160.

ODUM, E.; BARRETT, G. W. - **Fundamentals of Ecology.** [S.l.] : Cengage Learning, Inc. (2004). ISBN 978-0534420666.

OLSON, David M.; DINERSTEIN, Eric; KASSEM, Kenneth R. - Terrestrial ecoregions of the world: a new map of life on Earth. **BioScience**. ISSN 0006-3568. 51:11 (2001) 933. doi: 10.1641/0006-3568(2001)051[0933:TEOTWA]2.0.CO;2.

PASK, JD - The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. **Global Change. (**2012) 1231–1238. doi: 10.1111/j.1365-2486.2011.02622.x.

PEARMAN, P. B.; GARNER, T. W. J. - Susceptibility of Italian agile frog populations to an emerging Ranavirus parallels population genetic diversity. **Ecology Letters**. 8:2005) 401–408.

PEÑA-CASTRO, Julián Mario; MARTINEZ-JERONIMO, Fernando; ESPARZA-GARCIA, Fernando; CANIZARES-VILLANUEVA, Rosa Olivia - Phenotypic plasticity in *Scenedesmus incrassatulus* (Chlorophyceae) in response to heavy metals stress. **Chemosphere**. ISSN 00456535. 57:11 (2004) 1629–1636. doi: 10.1016/j.chemosphere.2004.06.041.

PEREIRA, Ruth; RIBEIRO, Rui; GONÇALVES, Fernando - Plan for an integrated human and environmental risk assessment in S. Domingos Mine area (Portugal). **Human and Ecological Risk Assessment: An International Journal**. 10 (2004) 543–578.

PIERCE, BA.; SIKAND, N. - Variation in acid tolerance of Connecticut wood frogs: genetic and maternal effects. **Canadian Journal of Zoology**. 63:1985) 1647–1651.

PÖLKKI, Mari; KANGASSALO, Katariina; RANTALA, Markus J. - Transgenerational effects of heavy metal pollution on immune defense of the blow fly *Protophormia terraenovae*. **PLoS ONE**. ISSN 19326203. 7:6 (2012). doi: 10.1371/journal.pone.0038832.

POUGH, F. H.; KAMEL, S. - Post-metamorphic change in activity metabolism of anurans in relation to life history. **Oecologia**. 65:1984) 138–144.

POUNDS, A. J.; CRUMP, M. L. - Amphibian declines and climate disturbance: the case of the Golden Toad and the Harlequin Frog. **Conservation biology**. 8:1 (1994) 72–85. doi: 10.1046/j.1523-1739.1994.08010072.x.

POUNDS, J.Alan; BUSTAMANTE, Martín R; YOUNG, Bruce E - Widespread amphibian extinctions from epidemic disease driven by global warming. **Nature**. ISSN 1476-4687. 439:7073 (2006) 161–7. doi: 10.1038/nature04246.

PURVIS, A.; HECTOR, A. - Getting the measure of biodiversity. **Nature**. 415 (2000) 212–219.

PYRON, R.Alexander; WIENS, John J. - A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. **Molecular phylogenetics and evolution**. ISSN 1095-9513. 61:2 (2011) 543–83. doi: 10.1016/j.ympev.2011.06.012.

RAINBOW, P. S. - Trace metal concentrations in aquatic invertebrates: why and so what? **Environmental Pollution**. 120 (2002) 497–507.

RAINBOW, P. S.; SCOTT, A. G. - Two heavy metal-binding proteins in the midgut gland of the crab *Carcinus maenas*. **Mar.Biol.** 55 (1979) 143–150.

RAINBOW, Philip S. - Trace metal bioaccumulation: models, metabolic availability and toxicity. **Environment international**. ISSN 0160-4120. 33:4 (2007) 576–82. doi: 10.1016/j.envint.2006.05.007.

RASANEN, K.; LAURILA, A.; MERILA, J. - Geographic variation in acid stress tolerance of the moor frog, *Rana arvalis* I. Local adaptation. **Evolution**. 57 (2003) 352–362.

RAVEN, P. H.; JOHNSON, G. B. - **Biology**. 10th. ed. [S.l.] : McGraw-Hill, (2014).

REGLERO, M. M.; MONSALVE-GONZALEZ, L.; TAGGART, M. A.; MATEO, R. - Transfer of metals to plants and red deer in an old lead mining area in Spain. **Science of the Total Environment**. ISSN 00489697. 406:1–2 (2008) 287–

297. doi: 10.1016/j.scitotenv.2008.06.001.

RELYEA, Rick A; DIECKS, Nicole - An unforeseen chain of events: lethal effects of pesticides on frogs at sublethal concentrations. **Ecological Applications**. ISSN 1051-0761. 18:7 (2008) 1728–42.

RIBEIRO, Rui; LOPES, Isabel - Contaminant driven genetic erosion and associated hypotheses on alleles loss, reduced population growth rate and increased susceptibility to future stressors: an essay. **Ecotoxicology (London, England)**. ISSN 1573-3017. 22:5 (2013) 889–99. doi: 10.1007/s10646-013-1070-0.

ROARK, Shaun; BROWN, Karen - Effects of Metal Contamination From Mine Tailings on Allozyme Distributions of Populations of Great Plains Fishes. **Environmental Toxicology and Chemistry**. ISSN 07307268. 15:6 (1996) 921–927. doi: 10.1897/1551-5028(1996)015<0921:EOMCFM>2.3.CO;2.

RODRÍGUEZ, L.; RUIZ, E.; ALONSO-AZCARATE, J.; RINCON, J. - Heavy metal distribution and chemical speciation in tailings and soils around a Pb-Zn mine in Spain. **Journal of Environmental Management**. ISSN 03014797. 90:2 (2009) 1106–1116. doi: 10.1016/j.jenvman.2008.04.007.

ROUSE, J. D.; BISHOP, C. A.; SRUGER, J. - Nitrogen pollution: an assessment of its threat to amphibian survival. **Environ. Health Perspect.** 107 (1999) 799–803.

SALA, OE; CHAPIN, FS; ARMESTO, JJ - Global biodiversity scenarios for the year 2100. **Science**. 287:March (2000) 1770–1774.

SANTO, Christophe Espírito; VASVONCELO MORAIS, Paula; GRASS, Gregor - Isolation and characterization of bacteria resistant to metallic copper surfaces. **Applied and environmental microbiology.** . ISSN 1098-5336. 76:5 (2010) 1341–8. doi: 10.1128/AEM.01952-09.

SANTORO, A. - Bioaccumulation of Heavy Metals by Aquatic Macroinvertebrates Along the Basento River in the South of Italy. **Water, Air, and Soil Pollution**. ISSN 0049-6979. 201:1–4 (2008) 19–31. doi: 10.1007/s11270-008-9923-5.

SCHEINER, S. M. - Genetics and evolution of phenotypic plasticity. **Annual Review of Ecology and Systematics**. 24 (1993) 35–68.

SHAW, A. J. - The evolution of heavy metal tolerance in plants: adaptations, limits, and costs. Em FORBES, V. E. (Ed.) - **Genetics and ecotoxicology.** [S.l.] : Taylor and Francis, Philadelphia (1999) p. 9–30.

SILLERO, N.; RIBEIRO, R. - Reproduction of Pelophylax perezi in brackish water in Porto (Portugal). **Herpetology Notes**. 3 (2010) 337–340.

SIMBERLOFF, Daniel - Flagships, umbrellas, and keystones: Is single-species management passé in the landscape era? **Biological Conservation**. 83:3 (1998) 247–257.

SMITH, M.Alex; GREEN, David M. - Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? **Ecography**. 28:1 (2005) 110–128.

SOBRAL, Olímpia; MARIN-MORALES, Maria Aparecida; RIBEIRO, Rui - Could contaminant induced mutations lead to a genetic diversity overestimation? **Ecotoxicology**. ISSN 09639292. 22:5 (2013) 838–846. doi: 10.1007/s10646-013-1079-4.

SOUSA, T. - Aerobic uranium immobilization by Rhodanobacter A2-61 through formation of intracellular uranium–phosphate complexes. **Metallomics**. 5 (2013) 390–397. doi: 10.1039/C3MT00052D

SPARLING, D. W.; T.P., Lowe. - Metal concentrations of tadpoles in experimental ponds. **Evironmental pollution.** 91:2 (1996) 149–159. doi: 10.1016/0269-7491(95)00057-7.

SPARLING, DW; LINDER, G; BISHOP, CA; KREST, SK - Ecotoxicology of amphibians and reptiles. (2010). ISBN 9781420064162 - 944 Pages - CRC Press.

STEVENSON, LA; ALFORD, RA; BELL, SC; ROZNIK, EA - Variation in thermal performance of a widespread pathogen, the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. **PloSone**. 8:9 (2013) 1–14. doi: 10.1371/journal.pone.0073830.

STRAALEN N.M., VAN; TIMMERMANS M.J.T.N. - Genetic variation in toxicants-stressed populations: An evaluation of the «Genatic Erosion» Hypothesis (2002).

STUART, SN; CHANSON, JS; COX, NA - Status and trends of amphibian declines and extinctions worldwide. **Science**. 306 (2004) 1783–1786.

TODD, Brian D. et al. - Aquatic and terrestrial stressors in amphibians: a test of the double jeopardy hypothesis based on maternally and trophically derived contaminants. **Environmental toxicology and chemistry / SETAC.** ISSN 1552-8618. 30:10 (2011) 2277–84. doi: 10.1002/etc.617.

US-EPA - **Short term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, EPA/600/4-89/001.** Cincinnati. [s.n.]

United Nations Conference on Environment and Development. **Agenda 21, Rio Declaration, Forest Principles.** New York: United Nations. (1992).

VIA, S.; LANDE, R. - Genotype-environment interaction and the evolution of phenotypic plasticity. **Evolution**. 39:3 (1985) 502–522.

VIA, Sara; GOMULKIEWICZ, R.; JONG, G. De - Adaptive phenotypic plasticity: consensus and controversy. **TREE**. 5 (1995).

VINEBROOKE, R. D.; COTTINGHAM, K.L.; NORBERG, J.; SCHEFFER, M.; DODSON, S.I.; MABERLY, S.C.; SOMMER, U. - Impacts of multiple stressors on biodiversity and ecosystem functioning: the role of species co-tolerance. **Oikos**. 104 (2004) 451–457.

WAH CHU, K.; CHOW, K. L. - Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. **Aquatic Toxicology**. 61 (2002) 53–64.

WEITERE, Markus; TAUTZ, Diethard; NEUMANN, Dietrich; STEINFARTZ, Sebastian - Adaptive divergence vs. environmental plasticity: tracing local genetic adaptation of metamorphosis traits in salamanders. **Molecular ecology**. ISSN 0962-1083. 13:6 (2004) 1665–77. doi: 10.1111/j.1365-294X.2004.02155.x.

WIEGAND, Thorsten; REVILLA, Eloy; MOLONEY, Kirk A. - Effects of habitat loss and fragmentation on population dynamics. **Conservation Biology**. ISSN 08888892. 19:1 (2005) 108–121. doi: 10.1111/j.1523-1739.2005.00208.x.

WILBUR, H. M. - Complex life cycles. **Annual Review of Ecological Systems**. 11 (1980) 67–93.

WILBUR, H. M.; COLLINS, J. P. - Ecological aspects of amphibian metamorphosis. **Science**. 182:1973) 1305–1314.

WILCOVE, D. S.; ROTHSTEIN, D.; DUBOW, J.; PHILLIPS, A.; LOSOS, E. - Quantifying threats to imperiled species in the United States. **Bioscience**. 48:8 (1998) 607–615. doi: 10.2307/1313420.

WILEY, E. O.; LEIBERMAN, B. S. - **Phylogenetics: Theory and Practice of Phylogenetic Systematics.** [S.l.] Wiley-Blackwell (2011). ISBN 978-0470905968.

WOODHAMS, Douglas C.; BLETZ, Molly; KUENEMAN, Jordan; McKENZIE, Valerie - Managing Amphibian Disease with Skin Microbiota. **Trends in Microbiology**. ISSN 0966842X. 24:3 (2016) 161–164. doi: 10.1016/j.tim.2015.12.010.

WORM, B.; BARBIER, E.B.; BEAUMONT, N.; WATSON, R. - Impacts of biodiversity loss on ocean ecosystem services. **Science**. 304:5008 (2006) 787–790. doi: DOI: 10.1126/science.1132294.

WRIGHT, David A. - Trace metal and major ion interactions in aquatic animals. **Marine Pollution Bulletin**. ISSN

0025326X. 31:1–3 (1995) 8–18. doi: 10.1016/0025-326X(95)00036-M.

YOUNG, Leah Bendell; HARVEY, Harold H. - Metal concentrations in crayfish tissues in relation to lake pH and metal concentrations in water and sediments. **Canadian Journal of Zoology**. ISSN 0008-4301. 69:4 (1991) 1076–1082. doi: 10.1139/z91-152.

YUE, Cai-Feng; WANG, Ting-Ting; WANG, Yu-Feng; PENG, Yu - Effect of combined photoperiod, water calcium concentration and pH on survival, growth, and moulting of juvenile crayfish (*Procambarus clarkii*) cultured under laboratory conditions. **Aquaculture Research**. ISSN 1355557X. 40:11 (2009) 1243–1250. doi: 10.1111/j.1365-2109.2009.02216.x.

ZOCCHE, J. J.; DA SILVA, L.A.; DAMIANI, A.P. - Heavy-metal content and oxidative damage in Hypsiboas faber: the impact of coal-mining pollutants on amphibians. **Archives of Environmental Contamination and Toxicology.** 66:1 (2014) 69–77. doi: 10.1007/s00244-013-9949-6.

CHAPTER 2.

Microevolution due to pollution in amphibians: a review on the genetic erosion hypothesis.

Microevolution due to pollution in amphibians: a review on the genetic erosion hypothesis.

E. Fasola[a], R. Ribeiro[b], I. Lopes[a]

[a] Department of Biology & CESAM (Centro de Estudos do Ambiente e do Mar), University of Aveiro, Campus de Santiago, 3810-193, Aveiro, Portugal.

[b] CFE – Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal.

Published by Environmental pollution, 204 (2015) 181-190, doi: 10.1016/j.envpol.2015.04.027

Abstract

The loss of genetic diversity, due to exposure to chemical contamination (genetic erosion), is a major threat to population viability. Genetic erosion is the loss of genotypes determining the value of a specific trait or set of traits. Almost a third of the known amphibian species is considered to be endangered and a decrease of genetic variability can push them to the verge of extinction. This review indicates that loss of genetic variation due to chemical contamination has effects on: 1) fitness, 2) environmental plasticity, 3) co-tolerance mechanisms, 4) trade-off mechanisms, and 5) tolerance to pathogens in amphibian populations.

Keywords

Genetic variability, fitness, environmental plasticity, co-tolerance, trade-off.

Introduction

Populations genetic structure results from past events and ongoing evolutionary processes (Carvalho and Brito, 2011; Chen *et al.*, 2012; Dong et al. 2012; Jehle and Arntzen, 2002; Teacher *et al.* 2009; Veith *et al.*, 2012; Vences *et al.*, 2013). Quaternary climatic oscillations and glaciations had profound effects on species distribution, causing severe fragmentation of populations (Carvalho and Brito, 2011; Chen *et al.*, 2012; Dong *et al.* 2012; Jehle and Arntzen, 2002; Teacher *et al.* 2009; Veith *et al.*, 2012; Vences *et al.*, 2013). Most of the species in temperate regions, of northern hemisphere, were displaced southwards reaching unfrosted refugees. These areas, relatively unaltered, were important

to shape the genetic structure of species and often conserved high levels of genetic diversity. Instead, populations established in recently occupied areas have reduced genetic diversity because of repeated bottlenecking during expansion, losing genetic variation by founder events (Carvalho and Brito, 2011; Chen *et al.*, 2012; Dong *et al.* 2012; Jehle and Arntzen, 2002; Teacher *et al.* 2009; Veith *et al.*, 2012). Present distributions result from these historical events and are often explained by restricted gene flow. This may be a distinctive feature of some taxa, including amphibians, that have low individual vagility (Vences *et al.*, 2013; Zeisset and Beebee, 2008). As with all other microevolutionary factors, also rates of genetic drift and gene flow can be altered by human pressure. Assessing the genetic diversity of populations is important for planning conservation strategies, especially for endangered species, as genetic variability is one of the levels of biological diversity requiring conservation (CBD, 2013; Hoban *et al.*, 2013; Luquet *et al.*, 2013; Medina *et al.* 2007). In this framework, The Convention on Biological Diversity promotes the conservation of genetic diversity by introducing the Aichi Targets: Target 13 states that countries, which signed the treaty must implement policies to minimize genetic erosion and safeguard populations genetic diversity (CBD, 2013). An understanding of past and ongoing status of populations genetic diversity is crucial because the maintenance of genetic variability prevents loss of the evolutionary potential of species (Bickham, 2011; Bijlsma and Loeschcke, 2012; Carvalho and Brito, 2011; Chen *et al.*, 2012; Coutellec and Barata, 2011; Lopes *et al.*, 2009; Nowak *et al.* 2009; O'Brien and Allentoft, 2010; Ribeiro and Lopes, 2013). Discriminate whether populations genetic structure resulted from the effect of recent demographic and environmental pressures or older historical influence is fundamental: conservation measures can only act on the present conditions (Chen *et al.*, 2012). Here, first we specify that the words: "stressor", "stress response" and "stress" will be used in this article in the sense introduced by Schulte (Schulte, 2014): "Stressors are environmental factors that cause stress. Organisms respond to stressors by mounting a stress response" (Schulte, 2014). We also assume that individuals experience different degree of stress according to both the kind and intensity of stressor and the organism that is experiencing it (Bijlsma and Loeschcke, 2005; Schulte, 2014). The individual sensitivity to a stressor is the result of its past experience with that particular stressor and of the evolutionary history of its species (Bijlsma and Loeschcke, 2005, 2012; Schulte, 2014). Mitigating ongoing toxicant-induced environmental stress should be a central point for conservation practices, because there is some evidence that environmental pollution is exerting impacts on genetic diversity of exposed populations through the occurrence of genetic erosion (Bickham, 2011; Bijlsma and Loeschcke, 2012; Coutellec and Barata, 2011; Lopes *et al.*, 2009; Luquet *et al.*, 2011, 2011; Medina *et al.* 2007; Nowak *et al.* 2009; O'Brien and Allentoft, 2010; Ribeiro and Lopes, 2013). According to Van Straalen and Timmermans (2002), "genetic erosion" is the loss of genotypes determining the

value of a specific trait or set of traits. Genetic erosion can lead to: loss of alleles in the population, reduction of population growth rate and increase of the susceptibility to further genetic erosion due to future exposure to other stressors. The acquisition of an increased tolerance in a natural population can occur through three mechanisms: 1) environmentally induced physiological adjustments in individuals (acclimation) (Chi-Shiun *et al.*, 2014; Maxwell *et al.*, 2014) (which does not cause shifts in the genetic structure of the population); 2) introduction of more tolerant genotypes by immigration and/or spread of tolerance genes due to mutation or immigration (which can alter population genetic structure) (Van Straalen and Roelofs, 2011); 3) loss of sensitive individuals (also by emigration), remaining only the most tolerant, leading to a reduction in the populations genetic variability (Forbes and Calow, 1996; Van Straalen *et al.* 2011). Population alleles frequencies will shift in scenarios 2 and 3; with a microevolutionary outcome; in these cases tolerance is determined genetically. A way for researchers to distinguish between the two microevolutionary scenarios could be to establish if sensitive genotypes are absent at the impacted site and tolerant genotypes are present at both the reference and impacted site. Therefore microevolution at the impacted site should have been caused by genetic erosion; not through the appearance of tolerance genes (Medina *et al.* 2007; Ribeiro and Lopes, 2013). Besides being caused by this contaminant-driven directional or stabilizing (in the case of heterosis) modes of selection, genetic erosion can also result from contaminant-driven random genetic drift, specifically bottlenecking (also through habitat fragmentation), which may lead to inbreeding. The evolutionary theory underlying the process of genetic erosion was recently discussed (Ribeiro and Lopes, 2013).

Moving the focus on amphibians

This review focus on contaminant-driven genetic erosion in amphibians but it is useful to start with a broader view on the class Amphibia, before going into detailed discussions. Amphibians are found on all continents (except Antarctica) with more than 7000 known species (Beebee, 2005; IUCN, 2013; Pyron and Wiens, 2011). The present divergence in amphibian species was mainly caused by migration history of this taxon (Beebee, 2005; Litvinchuk *et al.*, 2012; Nuñez *et al.*, 2011; Tingley and Dubey, 2012; Vences *et al.*, 2013). Among all vertebrates, amphibians exhibit the highest degree of population subdivision measured by allozyme and microsatellites markers (Beebee, 2005; Blaustein and Bancroft, 2007; Jehle and Arntzen, 2002; Nuñez *et al.*, 2011). Their populations are often very genetically differentiated, over relatively short geographical distances, while maintaining clear markers of historical events that generated current species distributions (Jehle and Arntzen, 2002; Nuñez *et al.*, 2011; Oza *et al.*, 2012; Vences *et al.*, 2013; Zeisset and Beebee, 2008).

Amphibians are very different from highly dispersing species (e.g. birds and flying insects) (Zeisset and Beebee, 2008); though this comparison is made between amphibians and flying animals, we should not underestimate their land displacement capabilities of dispersal (Green, 2003; Hayes *et al.* 2001; Smith and Green, 2005; Stebbins and Cohen, 1995; Tunner, 1992). Hence their "relatively low" mobility makes them ideal for studying major barriers to dispersal, both ancient and modern (Beebee, 2005). As a consequence, a complete conservation effort must take into account present menaces to amphibians' population genetic variability alongside with their postglacial history. This is crucial for amphibians as their pond breeding assemblages commonly have an effective size (N_e) <100 (Beebee, 2005; Beebee and Griffiths, 2005; Green, 2003; Laan and Verboom, 1990). Indeed, such small effective size (N_e), compared with census size (N_c), makes them highly susceptible to genetic depletion (Beebee and Griffiths, 2005; Ficetola *et al.*, 2010; Hedrick and Kalinowski, 2000; Hedrick, 2001; Laan and Verboom, 1990). Most amphibian species use both aquatic and terrestrial habitats; showing very different feeding habits and physiologies during their life stages (Arnold and Ovenden, 2002; Laudet, 2011; Ortiz-Santaliestra *et al.*, 2006; Pough e Kamel, 1984; Sparling *et al.*, 2010; Wilbur, 1980; Wilbur and Collins, 1973). This makes them susceptible to a wide range of stressors, thus being considered to be highly sensitive to environmental perturbations (Blaustein and Bancroft, 2007). Indeed amphibians are currently the most globally threatened group of vertebrates (Alford and Richards, 2007; Barinaga, 1990; Beebee and Griffiths, 2005; Blaustein, 1994; Blaustein and Kiesecker, 2002; Blaustein and Bancroft, 2007; Blaustein *et al.* 1994; Collins e Storfer, 2003; Drost and Fellers, 1996; Green, 2003; Hoffmann *et al.* 2010; Houlahan *et al.*, 2000; Kiesecker *et al.* 2001; Pechman and Wilbur, 1994; Pounds *et al.*, 2006; Pyron and Wiens, 2011; Storfer, 2003; Wake, 1991): Approximately 40% of all amphibian species are declining and almost 30% are listed in IUCN red list as "critically endangered", endangered" or "vulnerable" (IUCN, 2013). Habitat destruction, climate change, diseases, introduction of allochthonous species, pollution, and environmental acidification have all been described as important factors responsible for this global decline (Alford and Richards, 2007; Andren *et al.* 1989; Beebee, 1995; Beebee *et al.*, 1990; Beebee and Griffiths, 2005; Berger, 1989; Blaustein and Wake, 1990; Blaustein and Kiesecker, 2002; Blaustein and Bancroft, 2007; Carey and Bryant, 1995; Carr and Fahrig, 2001; Collins and Storfer, 2003; Corn and Vertucci, 1992; Davidson *et al.* 2002; Dunson *et al.* 1992; Fedorenkova and Vonk, 2012; Freda and Dunson, 1986; Gibbs *et al.* 2005; Ireland, 1991; Kiesecker *et al.* 2001; Knutson *et al.*, 1999; Maiorano *et al.*, 2013; Ortiz *et al.*, 2004; Pechman and Wilbur, 1994; Rasanen *et al.* 2003; Stuart, Chanson and Cox, 2004; Vatnick *et al.*, 1999; Vertucci and Corn, 1996; Wake and Vredenburg, 2008; Warner and Dunson, 1999; Whiteman *et al.* 1995; Wyman and Hawksley-Lescault, 1987). Despite habitat destruction being assumed to be a dominant cause for amphibians' populations decline (Barinaga,

1990; Blaustein and Bancroft, 2007; Carr and Fahrig, 2001; Gibbs, Whiteleather and Schueler, 2005; Green, 2003; Knutson *et al.*, 1999; Stuart *et al.* 2004), many other stressors can significantly impact their populations (Alford and Richards, 2007; Blaustein and Kiesecker, 2002; Blaustein and Bancroft, 2007; Blaustein *et al.*, 1994; Collins and Storfer, 2003; O'Brien and Allentoft, 2010). For example, acidification is a serious problem in habitats suitable for amphibians (Blem and Blem, 1989; Clark, 1986; Cook, 1983; Dale *et al.*, 1985; Dunson and Connell, 1982; Fioramonti *et al.*, 1997; Freda, 1986; Hangartner, 2012; Pierce, 1985; Pierce and Sikand, 1985; Pough, 1976; Räsänen *et al.*, 2005; Rowe *et al.*, 1992; Rowe and Freda, 2000) as well as increased UV radiation (Alton *et al.*, 2010; Belden and Blaustein, 2002; Blaustein *et al.*, 1994; Blaustein *et al.*, 2008; Blaustein and Kiesecker, 2002; Blaustein *et al.*, 2003; Blaustein and Bancroft, 2007; Garcia *et al.*, 2004; Langhelle *et al.*, 1999; Licht, 2003; O'Brien and Allentoft, 2010; Pahkala *et al.*, 2000; Uitregt *et al.*, 2007). Moreover, many of these habitats suffer pollution from nitrogen compounds, pesticides, herbicides, and metals (Adlassnig *et al.*, 2013; Allran and Karasov, 2001; Arco *et al.*, 1999; Baker and Waights, 1993, 1994; Beattie and Tyler-Jones, 1993; Berger, 1989; Boone *et al.* 2001; Boone and Semlitsch, 2002; Bridges, 2000; Britson and Threlkeld, 1998; Brühl *et al.*, 2013; Chen *et al.*, 2006, 2007, 2009; Clark *et al.*, 1998; Diana *et al.*, 2000; Ferguson and Gilbert, 1967; Freda, 1991; Gillardin *et al.*, 2009; Goyer and Clarkson, 2001; Hall and Henry, 1992; Hayes *et al.*, 2002; Hecnar, 1995; Hopkins *et al.*, 2000; Huey and Beitinger, 1980; James *et al.*, 2004; Jung and Jagoe, 1995; Karasov *et al.*, 2005; Linder and Grillitsch, 2000; Marco and Blaustein, 1998; Ortiz *et al.*, 2004; Ortiz-Santaliestra *et al.*, 2005, 2006, 2007; Owe *et al*, 1998; Rouse *et al.*, 1999; Rowe, Hopkins and Coffman, 2001; Rowe *et al.*, 1998; Sato *et al.*, 2013; Schuytema and Nebeker, 1999, 1999; Snodgrass *et al.*, 2004; Watt and Jarvis, 1997; Watt and Oldham, 1995; Xu and Oldham, 1997). Direct chemical application and runoff can be causes of stress on critical moments of amphibians life, like metamorphosis: emerging from the aquatic larval habitat to the terrestrial one is crucial for juveniles to enter next year breeding adult population (Berven, 1990; Semlitsch *et al.*, 1996; Semlitsch *et al.*, 2000). Bridges and Semlitsch (2000) suggested that one the explanation underlying global amphibians decline can be populations' divergence of genetically determined tolerance to chemicals (Bridges and Semlitsch, 2000; Semlitsch *et al*. 2000). Finally, an important concern is the spread of *Batrachochytrium dendrobatidis* infection that is threatening many amphibian populations (Baláž *et al.*, 2013; Bielby *et al.*, 2013; Blaustein *et al.*, 1994; Blaustein and Bancroft, 2007; Dinudom *et al.*, 2012; Liu, 2011; Mackie and Levinton, 2010; McKenzie and Peterson, 2012; Pounds *et al.*, 2006; Stevenson *et al.*, 2013; Stuart *et al.*, 2004). This fungus causes lethargic behaviour and skin sloughing, and in heavily infected individuals can inhibit electrical heart functions, causing cardiac arrest (Dinudom *et al.*, 2012).

Amphibians declining on a global scale, seem especially prone to genetic erosion processes. Semlitsch and colleagues (2000), noted how variation in genetically determined tolerance to chemicals and in fitness traits allow amphibian populations to persist and reproduce in contaminated environments (Semlitsch *et al.,* 2000). Decreased genetic variation can lead to reduced fitness and lack of adaptability to a changing environment (Willi *et al.,* 2006). However, few studies on the effects of contaminant-driven genetic erosion on amphibians have been carried out until the present; nevertheless, there is some evidence of its possible detrimental effects through the following mechanisms: 1) reduction of fitness, 2) reduced environmental plasticity, 3) absence of protective co-tolerance effects, 4) expression of tradeoffs between fitness and genetically determined tolerance mechanisms, 5) increased susceptibility to pathogens.

Genetic variability and reduced fitness

Genetic-fitness-correlations (GFCs), sensu O'Brien and Allentoft (2010) suggest that individuals genetic variability should be positively correlated with a higher fitness. GFCs are often documented in some published studies, although not always clearly (Hedrick and Kalinowski, 2000; Luquet *et al.*, 2011; O'Brien and Allentoft, 2010; Reed and Frankham, 2003). Fitness (measured as the total number of offspring reaching maturity) is difficult to measure in the field, for animals with, in general, external fertilization, like anuran amphibians. Because of the difficulties in tracking each individual throughout its lifetime, reproductive lifespan and offspring survival are technically very difficult to measure in nature (Lesbarrères *et al.*, 2005; Schulte, 2014). However, indicators of fitness (less accurate but easy to measure) are often used as fitness proxies (Schulte, 2014). For example, the reproductive output (the number or size of offspring produced per mating event) is used as a proxy for total reproductive success (Gibbons e McCarthy, 1986; Lesbarrères *et al.*, 2005; Luquet *et al.*, 2013). Indeed, it is possible that traits such as lifespan and fecundity may be positively correlated with fitness in most organisms (Merilä and Sheldon, 1999). In amphibians, links between tadpole and adult fitness are strong. Large size at metamorphosis is positively associated with survival and fecundity of adults (Beck and Congdon, 2000; Berven, 2013; Berven and Douglas, 1983; Miaud *et al.*, 1999; Scott, 1994; Semlitsch *et al.*, 1988; Smith, 1987; Wilbur, 1980; Wilbur and Collins, 1973), because performance during adult life is influenced by carryover effects from conditions experienced during early development (Beck and Congdon, 2000; Earl and Semlitsch, 2013; Räsänen *et al*., 2002; Scott, 1994; Semlitsch *et al*., 1988; Smith, 1987; Wilbur, 1980; Wilbur and Collins, 1973). Hence, fitness in amphibians can be measured by age, growth rate, survival, or reproductive success (Beck and Congdon, 2000; Berven, 2013; Luquet *et al.*, 2011, 2013; Schmeller *et al*., 2007). After

establishing reliable fitness proxies, the relationship between fitness and genetic variability has been investigated through the associations between marker-based measures of genetic variability and fitness-related traits (Allendorf and Leary, 1986; David, 1998; Lesbarrères *et al.*, 2005; Luquet *et al.*, 2011, 2013). GFCs, assuming differences in inbreeding coefficient, result from effects of homozygosity at genome-wide distributed loci (general effect hypothesis), therefore a link between genetic variability and reproductive success is possible (Hansson and Westerberg, 2002; Lesbarrères *et al.*, 2005), linking genetic diversity with mean populations fitness (Allendorf and Leary, 1986). Three hypotheses have been proposed to explain correlations between molecular markers heterozygosity and fitness: 1) multilocus GFC might result from selection acting directly at a particular loci; 2) linkage disequilibrium (e.g. in recently bottlenecked and expanded populations) may cause associations between the markers and fitness loci in the local chromosomal vicinity; 3) heterozygosity can reflect variation in the inbreeding coefficient and associate with fitness by effects of homozygosity at genome-wide distributed loci (Hansson and Westerberg, 2002) (heterozygosity may correlate with fitness, due to functional overdominance, associative overdominance, or heterosis). Lesbarrères et al. (2005), under the assumption that: (i) microsatellite variability reflects variability in genome wide heterozygosity and that (ii) the size and age at metamorphosis are positively related to fitness, predicted a relationship between these two life history traits and genetic variability in *Rana temporaria*. Those authors found a correlation between genetic variability and offspring weight in the wild. In amphibians offspring weight/size correlates positively with survival. Lesbarrères et al. (2005) concluded that larval survival is related to genetic variability. However, these results can be biased by: 1) high number of tested inbred individuals due to the sampling method used, or 2) linkage disequilibrium between molecular markers. Despite these possible weaknesses, recognized by Lesbarrères and collaborators, the authors of this review still agree with their conclusions: survival was found positively correlated to genetic variability, through tadpole size. Some authors observed a positive correlation between genetic diversity at microsatellite loci and fitness (Ficetola *et al.*, 2010; Ficetola *et al.*, 2007; Pearman and Garner, 2005). Ficetola et al. (2010) used microsatellite loci to detect significant variation in genetic variability in *Rana latastei*, and this variation strongly correlated with fitness (Ficetola *et al.*, 2010). Luquet et al. (2011) found positive correlations between microsatellites heterozygosity and larval performances in *Hyla arborea*. They also confirmed that the observed GFCs were not a consequence of maternal heterozygosity effects, but a true relationship between individual heterozygosity and fitness (Luquet *et al.*, 2011). However, later, the same authors did not found any correlation between reduced body size or reproductive effort and multilocus heterozygosity, in adults of *H. arborea* (Luquet *et al.*, 2013), but the lack of observed heterozygosity-fitness correlation was due to the lack of inbreeding (ie, heterozygosity did not vary

enough to show any relationship). Schmeller *et al*. (2007) investigated if longevity of *Phelophylax perezi*, in stressful environments, is positively related to allozyme heterozygosity. They found that frogs survival is linked to higher allozyme heterozygosity, which is assumed to increase adaptability to stressors. Furthermore, their results show that GFCs are more evident under stressful conditions (Cassel *et al.*, 2001). Given these examples, it is evident that in some amphibians species, heterozygous individuals show increased fitness in comparison to homozygous conspecifics. Hence, genetic erosion is clearly a threat to amphibians populations viability. It can reduce mean fitness, lead to increased inbreeding and, eventually, to genetic load (Allendorf and Leary, 1986; Hedrick and Kalinowski, 2000; Hedrick, 2001; Keller and Waller, 2002; Merilä and Sheldon, 1999; Reed and Frankham, 2003; Rowe and Beebee, 2003). Amphibians have often small effective population sizes, with few individuals contributing to the gene pool each mating season: they would be easily affected by inbreeding enhanced by genetic erosion, which could drive local populations to extinction.

Genetic variability and environmental plasticity

Local adaptation to new environmental conditions is cause of divergence and speciation (Dieckmann and Doebeli, 2004). An alternative option to cope with continuously changing environments is "phenotypic plasticity": the ability of a certain genotype to generate different phenotypes under changing environments (Buskirk *et al*., 1998; De Witt *et al*., 1998; Ghalambor *et al.*, 2007; Gomulkiewicz and Kirkpatrick, 1992; Maxwell *et al.*, 2014; Merila *et al.*, 2000; Pigliucci and Schlichting, 1998; Scheiner, 1993; Schulte, 2014; Sultan and Spencer, 2002; Via *et al*. 1995; Weitere *et al.*, 2004). An individual can face the effects of a stressor by using phenotypic plasticity (Schulte, 2014), in this case plasticity is a stress response, *sensu* Schulte (2014). Adaptive phenotypic responses can have great utility; especially for species living in a constantly shifting environment (De Witt *et al.*, 1998; Gomulkiewicz and Kirkpatrick, 1992; Pigliucci and Schlichting, 1998; Scheiner, 1993; Sultan and Spencer, 2002); because populations are affected both by natural or anthropic stressors (namely pollutants). Phenotypic plasticity helps individuals to cope with abrupt habitat variations because each genotype could be able to retain enough fitness (Scheiner, 1993; Sultan and Spencer, 2002; Travis, 1994). Hence, genetic erosion impacts on the expression, and associated costs, of plastic responses may be really harmful for populations viability (Bijlsma and Loeschcke, 2012; De Witt *et al.*, 1998; Gomulkiewicz and Kirkpatrick, 1992; Reed and Frankham, 2003; Weitere *et al.*, 2004). In the presence of a constant gene flow between populations, phenotypic plasticity of traits is promoted by heterogeneity of habitats, as the phenotype of each habitat is better adapted to the particular conditions of that environment (Gomez-Mestre and Tejedo, 2003; Rawson and Hilbish, 1991; Sultan

and Spencer, 2002). Furthermore, a plastic genotype will be promoted in metapopulations as long as there is even a low migration rates between subpopulations (Sultan and Spencer, 2002). On the other hand, if gene flow is absent, stressful conditions select for local adaptation rather than plastic responses (Gomez-Mestre and Tejedo, 2003; Rasanen *et al*., 2003; Rawson and Hilbish, 1991; Sultan and Spencer, 2002). Amphibians populations are mainly framed as metapopulations, characterized by low migration rates (Beebee, 2005; Beebee and Griffiths, 2005; Zeisset and Beebee, 2008). The model proposed by Sultan et al. (2002) demonstrated that metapopulation structure allows a genotype to be fixed in a certain subpopulation, even if it does not have the highest fitness at that site. They also suggested that plasticity can be very important to maintain fitness in metapopulations because even rare migration events could allow subpopulations to retain enough genetic variation for plastic responses (Sultan and Spencer, 2002). On the other hand, genetic erosion processes, under these conditions, are enhanced by reduced migration between demes in a metapopulation thus possibly reducing genetic variability for plasticity (Ghalambor *et al.*, 2007; Gomez-Mestre and Tejedo, 2003; Medina *et al.* 2007; Via *et al*., 1995), consequently lowering individuals plastic capabilities. Amphibians show a broad display of plastic responses: larvae of many species of anurans express phenotypic plasticity in: development, adaptive morphology and behavior (Berven and Douglas, 1983; Buskirk, 2002; Buskirk *et al*., 1998; Laurila *et al*., 2002; Luquet *et al*., 2011; Merila *et al*., 2000; Newman, 1988; Polcák and Gvozdík, 2014). For example, some anuran tadpoles are able to respond to pond drying by metamorphosing earlier (Laurila *et al*., 2002; Merila *et al*., 2000; Newman, 1988, 1992). Luquet et al. (2011) suggested that phenotypic plasticity can be maintained in *H. arborea* populations, impacted by genetic erosion, only with subsequential impairment of life history traits (reduced survival and lower developmental and growth rates). The relationship between plasticity and genetic erosion has not yet been clearly assessed. Anyway, phenotypic plasticity has a genetic basis (Via and Lande, 1985; Via *et al.*, 1995); genetic variation for plastic responses is commonly observed (Newman, 1988). Therefore, for some authors, phenotypic plasticity, for a character, should be seen as separated from the mean value, of the same character, which is under its genetic regulation, between different environments (Scheiner, 1993; Scheiner and Lyman, 1991; Via and Lande, 1985; Via, 1993; Via *et al.*, 1995). On the other hand, there are scholars who think that the mean phenotype for each different environmental condition, and the plasticity itself for that particular trait, are determined by the same loci (Via, 1993). Also, hypothesizing that in most of the cases the latter scenario would be true, we cannot neglect that natural selection is, at least sometimes capable to act on phenotypic plasticity itself (Via, 1993; Via *et al.*, 1995). Thus, genetic erosion may hamper plastic responses (Bijlsma and Loeschcke, 2012; Newman, 1988), but such effects should not be easily generalized, also when plasticity acts positively on fitness. The genetic architecture of

plasticity may differ from that of basic fitness traits (Gomulkiewicz and Kirkpatrick, 1992; Pigliucci and Schlichting, 1998; Rowe and Beebee, 2003; Scheiner, 1993). Hence, it is mandatory to acquire a better understanding of the genetic basis of phenotypic plasticity and the possible negative effects correlated with genetic erosion impacts.

Genetic variability and co-tolerance

A single-stressor perspective is inadequate because individuals, populations and ecosystems are threatened by multiple co-occurring stressors (Alton *et al.*, 2010; Beebee and Griffiths, 2005; Blaustein *et al.*, 2008; Blaustein and Kiesecker, 2002; Boone and Stacy, 2003; Boone and Semlitsch, 2002; Britson and Threlkeld, 1998; Darling *et al.*, 2010, 2013; Fioramonti *et al.*, 1997; Folt *et al.*, 1999; James *et al.*, 2004; Owe *et al.*, 1998; Pahkala *et al.*, 2002; Slocum and Mendelssohn, 2008; Threlkeld and Britson, 2000; Wah Chu and Chow, 2002), acting both directly and indirectly (Boone and Stacy, 2003; Horne and Dunson, 1995; Relyea and Diecks, 2008). Indeed, populations may be exposed to factors, that under normal conditions, do not cause their decline but, when more stressors are added, those populations may be forced beyond their tolerance limits and genetic erosion effects may be induced (Alton *et al.*, 2010; Beebee and Griffiths, 2005; Blaustein and Bancroft, 2007; Blaustein *et al*., 1994; Darling *et al.*, 2010; Medina *et al*., 2007; Ortiz-Santaliestra *et al.*, 2010; Pahkala *et al*., 2002; Ribeiro and Lopes, 2013; Sala *et al*., 2000; Santos *et al.*, 2013; Vinebrooke *et al.*, 2004). Combined effect of multiple co-occurring stressors may act synergistically or in an antagonistic way; in either cases, this is different from the simple sum (additivity) of each separate factor, hence, it would be unforeseen by single-stressor studies (Beebee and Griffiths, 2005; Blaustein *et al.*, 2008; Boone and Stacy, 2003; Folt *et al.*, 1999; Owe, Illis and Owbray, 1998; Pahkala *et al*., 2002; Threlkeld and Britson, 2000; Vinebrooke *et al.*, 2004; Wah Chu and Chow, 2002). When individuals of a population are, or have been, exposed to two or more different stressors (not necessarily at the same time), some of them can develop co-tolerance mechanisms. Those individuals will use the same metabolic detoxification pathways to face both stressors (this should lower metabolic costs). Co-tolerance may arise as: 1) specific tolerance mechanisms associated with previous exposure to both stressors, 2) generalist counteraction against the effects of many stressors (Cobbett and Goldsbrough, 2002; Coyle *et al.*, 2002; Medina *et al*., 2007; Ribeiro and Lopes, 2013). Co-tolerance was found in invertebrates (Brown, 1978; Langdon *et al.*, 1999; Langdon and Piearce, 2001; Lopes *et al*., 2005; Spurgeon and Hopkin, 2000) and in plants (Cobbett and Goldsbrough, 2002) for pollutants with the same mode of action, such as some different metal couples: copper/arsenic, copper/zinc, copper/lead. It is also generally accepted that decreasing genetic variability lowers the

adaptive potential towards multiple stressors (Medina *et al*., 2007; Ribeiro and Lopes, 2013; Semlitsch *et al*., 2000). So far, some studies investigated co-tolerance in amphibians, among others. They explored the interactions between: 1) osmotic stress and tolerance to pesticides in *P. perezi* (Ortiz-Santaliestra *et al.*, 2010), 2) osmotic stress and tolerance to metals in *P. perezi* (Santos *et al.*, 2013), 3) two pesticides in *Fejervarya limnocharis* (Nataraj and Krishnamurthy, 2012), 4) two herbicides and an insecticide in *Hyla versicolor* tadpoles (Mazanti *et al.*, 2003), 5) an herbicide atrazine and an insecticide in *Rana sphenocephala*, *Bufo americanus*, *Ambystoma texanum* and *Ambystoma maculatum* (Boone and Stacy, 2003), 6) pH and water hardness in *Ambystoma jeffersonianum* and *Rana sylvatica* (Horne and Dunson, 1995), proving that interactions in this complex system are mediated by all the abiotic components together as well as the physiology of the species. Others studies focused on increasing UV radiation and its correlation to: 1) higher susceptibility of *Bufo boreas* embryos to *Saprolegnia ferax* (Kiesecker and Blaustein, 1995; Kiesecker *et al*., 2001), 2) increased toxicity of insecticides in anurans larvae (Zaga *et al.*, 2008), 3) synergistic effects with nitrate fertilizers (Hatch and Blaustein, 2003), as well as nitrite compounds in *Bufo bufo* and *P. perezi* (Macìas et al., 2007) larval stages, 4) interactions with copper pollution in *Pseudacris crucifer* tadpoles (Baud and Beck, 2005), 5) synergisms with lower pH in embryos of *R. temporaria* (Pahkala *et al*., 2002), as well as interaction between increasing and lowering pH and triphenyltin pollution in parental species and hybrid of the *Pelophylax* complex (hybrid: *P. esculentus*, parental species used: *P. lessonae*, formerly *Rana* sinklepton *esculenta*) (Fioramonti *et al.*, 1997); in this study, Fioramonti et al. (Fioramonti *et al.*, 1997) demonstrated how the hybrid is less sensitive to the fungicide than its parental species; if this is due to the higher heterozygosity of *P. esculentus* is still to be evaluated, 6) finally, a triple synergic interaction between UV radiation with nitrate and low pH, which reduced performances of *Rana cascadae* (Hatch and Blaustein, 2000), showing how the presence of multiple factors was detrimental, while a single factor alone was not. All these works investigated the existence of co-tolerance; some of them explored its possible interaction with genetic depletion. Unfortunately, there is still a gap in our knowledge, which needs to be filled, to better understand these mechanisms.

Genetic variability and fitness trade-off costs

Trade-offs arise when the ability of an organism to perform in one ecological context comes at the expense of its abilities in other situations (Forbes and Calow, 1996; Gomez-Mestre and Tejedo, 2003; Huey and Slatkin, 1976; Mueller *et al*., 2012; Newman, 1988; Nicieza, 2000; Semlitsch *et al*., 2000; Sultan and Spencer, 2002). These processes can diminish the diffusion of tolerant genotypes in

pristine habitats and lower populations evolutionary capabilities (Semlitsch *et al*., 2000; Shaw, 1999). Organisms use many strategies to cope with pollution: 1) exclusion (e.g. many aquatic animals, like amphibians, secrete mucus onto exposed surfaces); 2) removal (toxicants actively pumped out); 3) detoxification, possibly followed by excretion (e.g. by sequestration or metabolic transformation); 4) repair of damage caused by toxicants. All these responses can be metabolically costly, leading to energetic trade-off costs (Calow, 1991; Forbes and Calow, 1996; Koehn and Bayne, 1989; Rowe *et al.*, 1998). For example, the production of mucus (on amphibians skin) may inhibit uptake of toxic chemicals but also have a substantial metabolic cost (i.e. individuals forced to produce more mucus could grow less than non exposed ones, due to different energy allocation) (Calow, 1991; Forbes and Calow, 1996). Individuals can bear trade-off costs only for a short period, after the exposure to a stressor, or during their entire lifetime. This depends upon their capacity to face that stressor: it can be constitutive or inducible. In the former case, detoxification mechanisms are maintained continuously at a high level; in the latter case, it is activated only immediately after the exposure (Calow, 1991; Cousteau *et al*., 2000; Forbes and Calow, 1996; Koehn and Bayne, 1989; Rowe *et al.*, 1998). These two strategies bear substantially different metabolic costs that should be higher in the former (except for a constitutive mechanism of detoxification, arisen due to evolutionary adaptation, which has the possibility not to bear trade-offs). Different genotypes occupy different positions on the trade-off curves between investment in producing stress responses and life history traits (depending on their metabolic efficiency). On the other hand, different genotypes may vary in their ability to shift to different points on the trade-off curve, depending upon stressful conditions (due to differential ability to cope with them) (Fisker *et al.*, 2011; Forbes and Calow, 1996; Koehn and Bayne, 1989). Thus, it is possible that costs of living in stressful environments, by trading off with other elements of the energy budget (that influence survival, growth and reproduction) can influence population dynamics (Forbes and Calow, 1996; Koehn and Bayne, 1989). Indeed, some studies have shown that microevolution due to pollution can bear fitness costs associated with the altered physiological processes enabling individuals to cope with the stress. These fitness costs can exert their effect on populations viability or reproductive processes; lowering effective size (N_e) or increasing inbreeding (Medina *et al*., 2007; Ribeiro and Lopes, 2013; Van Straalen and Timmermans, 2002). This scenario would be especially harmful for amphibians who often display low N_e (Beebee, 2005). A trade-off between tolerance to carbaryl and survival for tadpoles of *H. versicolor* (Semlitsch *et al.*, 2000) has been observed; on the other hand, another study found no trade-off between tolerance to salinity and fitness in *Bufo calamita* (Gomez-Mestre and Tejedo, 2003). Furthermore, in this former study (Semlitsch *et al*., 2000) a trade-off was detected only when *H. versicolor* tadpoles were raised at high density. Indeed Semlitsch showed how inducing more stressful conditions, by increasing

tadpoles density in his tanks, helped reveling the existence of the trade-off mechanism (Semlitsch *et al*., 2000). Competition between tadpoles, raised at high density, is not commonly added as an experimental factor. Possibly making standard experimental conditions not harsh enough to reveal the existence of trade-off. Thus further research is needed to clarify the trade-off mechanisms in amphibians populations under stressful conditions.

Genetic variability and tolerance to pathogens

Genetic variability has a role in increased tolerance to pathogens. Inbred populations could be relatively tolerant to one pathogen, but are likely to be susceptible to most other unrelated pathogens or to different strains of the same pathogen (Reed and Frankham, 2003; Savage and Zamudio, 2011). Furthermore, an allele that provides tolerance to an infectious disease could be detrimental when the pathogen is absent, because of pleiotropy associated with that allele (Sparling *et al.*, 2010). Heterozygosity is very important for the functioning of the immune system, in particular for the major histocompatibility complex (MHC) (Halverson *et al*., 2006; Savage and Zamudio, 2011), which is the most polymorphic genetic system ever described in vertebrates, and constitutes the most famous case of diversifying selection. Indeed, inbreeding and loss of genetic diversity decrease tolerance to pathogens in invertebrates (Spielman *et al.*, 2004) and vertebrates (Radwan *et al.*, 2010). Mostly, amphibians lay their eggs in water bodies and larvae are subjected to a number of waterborne pathogens (Blaustein and Bancroft, 2007). In this context Savage and Zamudio (2011) found that MHC heterozygous individual of *Rana yavapaiensis* show significant increased capability to face chytridiomycosis. The authors suggest that heterozygote advantage may be the general mechanism to increase chytridiomycosis tolerance in those amphibians populations. In another paper Luquet *et al*. (2012) hypothesized that the deleterious consequences of exposure to *B. dendrobatidis* would be more severe for *H. arborea* tadpoles from populations with lower genetic variability. Despite exposed tadpoles lacked infection, the authors concluded that genetic erosion does not affect the capacity to develop effective tolerance strategies but erodes the possibility to invest simultaneously in: tolerance to pathogen, increased tadpole growth rate and size at metamorphosis. However, these results could be due to genetic erosion on fitness traits might and not due to tolerance mechanisms. In another paper, Daum et al. (Daum *et al.*, 2012) showed that genetic heterogeneity can enhance the antimicrobial peptides (AMPs) immune defense capacity in hybrids of the *Pelophylax* complex (consisting of the hybrid *P. esculentus* and its parent species *P. lessonae* and *P. ridibundus*) (Arnold and Ovenden, 2002; Kuzmin *et al.*, 2009). Hence, the authors showed that, within stressful environment (with many parasites), heterosis (namely vigor of the hybrids) improves immune

function against *B. dendrobatidis*. Daum et al. (Daum *et al.*, 2012) thus proved the existence of a correlation between higher genetic variability and tolerance to pathogens. However this topic needs more studies to improve our knowledge especially as amphibians are now facing a global chytridiomycosis pandemic (Baláž *et al.*, 2013; Blaustein *et al.*, 1994; Hoffmann *et al.*, 2010; Liu, 2011; McKenzie and Peterson, 2012; Stuart *et al.*, 2004).

Conclusion

This review reports various studies investigating the detrimental effects, related to microevolutionary processes, able to decrease genetic diversity. However, research does not often focus on genetic erosion and its effects have been usually underestimated in amphibians. On the other hand, amphibians could be an ideal taxon, among vertebrates, to study genetic erosion mechanisms. They are facing a huge global decline. Their fragmented and endangered populations often naturally occur as highly inbred in a lowered genetic variability status. Hence, amphibians populations represent a model possibility to measure the impacts of pollution-induced decrease of genetic diversity (namely contaminant-driven genetic erosion). In the above sections, there are examples of studies correlating lower genetic diversity with decreased: 1) fitness, 2) environmental plasticity and 3) tolerance mechanisms towards pollution or pathogens. However, these correlations are still being debated as, due to contrasting results, a clear connection has not always been established. A possible reason for these contrasting findings, at least regarding amphibians, could hide in their population structure. Many researchers started their studies with the supposition of limited dispersal (causing isolation) as the reason for assumed metapopulation structures in amphibians (Stebbins and Cohen, 1995). However, this a priori assumption is often not correct (especially for anurans) (Smith and Green, 2005; Stebbins and Cohen, 1995; Hayes et al., 2001; Tunner, 1992). Therefore, there is the possibility that such studies could biased: instead of comparing two different populations, by assuming limited dispersal, they could actually sampling the same one. As a result, focused research is needed to disentangle the structure of microevolutionary processes caused by genetic erosion. This process is arising as one of the main factors accountable for biodiversity (namely genetic diversity) global loss in wild populations.

Acknowledgments

This study was funded by national funds (OE) through FCT/MEC and co-funded by Fundo Europeu de Desenvolvimento Regional-FEDER through COMPETE–Programa Operacional Factores de Competitividade (POFC) (projects PTDC/BIA-BIC/3488/2012 and PEst-C/MAR/LA0017/2013) and through a PhD scholarship to Emanuele Fasola (SFRH/BD/88955/2012), endorsed by FCT. We also thank Dr. Samikannu Iswarya, Dr. Claudia Wiegand and three more anonymous reviewers who helped us improving the quality of this paper.

References

ADLASSNIG, Wolfram; SASSMANN, Stefan; GRAWUNDER, Anja; PUSCHENREITER, Markus; HORVATH, Amadea; KOLLER-PEROUTKA, Marianne - Amphibians in metal-contaminated habitats. **Salamandra**. 49:3 (2013) 149–158.

ALFORD, Ross A.; RICHARDS, Stephen J. - Global amphibian declines: a problem in applied ecology. **Annual Review of Ecology and Systematics**. 30:1999 (2007) 133–165.

ALLENDORF, F. W.; LEARY, R. - Heterozygosity and fitness in natural populations of animals. In: **Conservation biology: the science of scarcity and diversity**. [S.l.] : Soule M.E. (ed). Conservation Biology. Sinauer: Sunderland, MA (1986) p. 75–76.

ALLRAN, J. W.; KARASOV, W. H. - Effects of atrazine on embryos, larvae, and adults of anuran amphibians. **Environmental Toxicology and Chemistry**. 20 (2001) 769–775.

ALTON, Lesley A.; WILSON, Robbie S.; FRANKLIN, Craig E. - Risk of predation enhances the lethal effects of UV-B in amphibians. **Global Change Biology**. ISSN 13541013. 16:2 (2010) 538–545. doi: 10.1111/j.1365-2486.2009.02010.x.

ANDREN, C.; MARDEN, M.; NILSSON, G. - Tolerance to low pH in a population of moorfrogs, *Rana arvalis*, from an acid and a neutral environment: a possible case of rapid evolutionary response to acidification. **Oikos**. 56 (1989) 215–223.

ARNOLD, Nicolas; OVENDEN, Denys - **Reptiles and Amphibians of Britain and Europe.** [S.l.] Collins; New edition (2002).

BAKER, J. M. R.; WAIGHTS, V. - The effect of sodium nitrate on the growth and survival of toad tadpoles (*Bufo bufo*) in the laboratory. **Herpetological Journal**. 3 (1993) 147–148.

BAKER, J. M. R.; WAIGHTS, V. - The effects of nitrate on tadpoles of the tree frog (*Litoria caerulea*). **Herpetological Journal**. 4 (1994) 106–108.

BALÁŽ, Vojtech; VOROS, Judit; IVIS, Petr; VOJAR, Jiri; HETTYEY, Attila; SOS, Endre; DANKOVICS, Róbert; JEHLE, Robert; CHRISTIANSEN, Ditte G; CLARE, Frances; FISHER, Matthew C; GARNER, Trenton W J; BIELBY, Jon. - Assessing Risk and Guidance on Monitoring of *Batrachochytrium dendrobatidis* in Europe through Identification of Taxonomic Selectivity of Infection. **Conservation biology : the journal of the Society for Conservation Biology**.

ISSN 1523-1739. (2013) 1–11. doi: 10.1111/cobi.12128.

BARINAGA, M. - Where have all the froggies gone? **Science**. 2 (1990) 1033–1034.

BAUD, D. R.; BECK, M. L. - Interactive effects of UV-B and copper on spring peeper tadpoles (*Pseudacris crucifer*). **Southeastern Naturalist**. 4 (2005) 15–22.

BEATTIE, R. C.; TYLER-JONES, R. - The effects of low pH and aluminum on breeding success in the frog *Rana temporaria*. **Journal of Erpethology**. 26 (1993) 353–360.

BECK, C. W.; CONGDON, J. D. - Effects of age and size at metamorphosis on performance and metabolic rates of Southern Toad, *Bufo terrestris*, metamorphs. **Functional Ecology**. 14 (2000) 32–38.

BEEBEE, T. J. C.; FLOWER, R.J.; STEVENSON, A.C.; PATRICK, S.T.; APPLEBY, P.G.; FLETCHER, C.; MARSH, C.; NATKANSKI, J.; RIPPEY, B.; BATTARBEE, R.W. - Decline of the natterjack toad *Bufo calamita* in Britain: palaeoecological, documentary and experimental evidence for breeding site acidification. **Biological Conservation**. 53 (1990) 1–20.

BEEBEE, T. J. C. - Amphibian breeding and climate. **Scientific correspondence, Nature.** 374:1995) 219–220.

BEEBEE, T. J. C. - Conservation genetics of amphibians. **Heredity**. ISSN 0018-067X. 95:6 (2005) 423–7. doi: 10.1038/sj.hdy.6800736.

BEEBEE, T. J. C.; GRIFFITHS, Richard A. - The amphibian decline crisis: A watershed for conservation biology? **Biological Conservation**. ISSN 00063207. 125:3 (2005) 271–285. doi: 10.1016/j.biocon.2005.04.009.

BELDEN, L. K.; BLAUSTEIN, A. R. - Exposure of red-legged frog embryos to ambient UV-B radiation in the field negatively affects larval growth and development. **Oecologia**. 130 (2002) 551–554.

BERGER, L. - Disappearance of amphibian larvae in the agricultural landscape. **Ecological International Bullettin**. 17 (1989) 65–73.

BERVEN, Keith A. - Factors affecting population flucuations in larval and adult stages of the wood frog (*Rana sylvatica*). **Ecology**. 71 (1990) 1599–1608.

BERVEN, Keith A. - Factors Affecting Population Fluctuations in Larval and Adult Stages of the Wood Frog (*Rana Sylvatica*). Published by : Ecological Society of America Stable URL : http://www.jstor.org/stable/1938295 . LA. 71:4 (2013) 1599–1608.

BERVEN, Keith A.; DOUGLAS, Gill E. - Interpreting geographic variation in life-history traits. **American Zoologist**. 23:1 (1983).

BICKHAM, JW - The four cornerstones of evolutionary toxicology. **Ecotoxicology**. (2011) 497–502. doi: 10.1007/s10646-011-0636-y.

BIELBY, J.; BOVERO, S.; ANGELINI, C.; FAVELLI, M.; GAZZANIGA, E.; PERKINS, M.; SOTGIU, G.; TESSA, G.; GARNER, T. W. J. - Geographic and taxonomic variation in *Batrachochytrium dendrobatidis* infection and transmission within a highly endemic amphibian community. **Diversity and Distributions**. ISSN 13669516. 19:9 (2013) 1153–1163. doi: 10.1111/ddi.12085.

BIJLSMA, R.; LOESCHCKE, V. - Environmental stress, adaptation and evolution: an overview. **Journal of evolutionary biology**. ISSN 1010-061X. 18:4 (2005) 744–9. doi: 10.1111/j.1420-9101.2005.00962.x.

BIJLSMA, R.; LOESCHCKE, Volker - Genetic erosion impedes adaptive responses to stressful environments. **Evolutionary Applications**. ISSN 17524571. 5:2 (2012) 117–129. doi: 10.1111/j.1752-4571.2011.00214.x.

BLAUSTEIN, A. R. - Chicken little or Nero's fiddle? A perspective on declining amphibian populations. **Herpetologica**. 50 (1994) 85–97.

BLAUSTEIN, A. R. HOFFMAN, P D; HOKIT, D G; KIESECKER, J M; WALLS, S C; HAYS, J B - UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines? **Proceedings of the National Academy of Sciences of the United States of America**. ISSN 0027-8424. 91:5 (1994) 1791–5.

BLAUSTEIN, A. R.; HOKIT, D. G.; O'HARA, R. K. - Pathogenic fungus contributes to amphibian losses in the Pacific Northwest. **Biological Conservation**. 67 (1994) 251–254.

BLAUSTEIN, A. R.; WAKE, D. B. - Declining amphibian populations: a global phenomenon? **Trends in ecology & evolution**. 5:1990) 203–204.

BLAUSTEIN, Andrew R.; BANCROFT, B. A.; BAKER, N. J. - A meta-analysis of the effects of ultraviolet B radiation and its synergistic interactions with pH, contaminants, and disease on amphibian survival. **Conservation Biology**. 22 (2008) 987–996.

BLAUSTEIN, Andrew R.; KIESECKER, Joseph M. - Complexity in conservation: lessons from the global decline of amphibian populations. **Ecology Letters**. 5 (2002) 597–608.

BLAUSTEIN, Andrew R.; ROMANSIC, John M.; KIESECKER, Joseph M. - Ultraviolet radiation , toxic chemicals and amphibian population declines. **Diversity and Distributions**. SPECIAL ISSUE : AMPHIBIAN DECLINES (2003) 123–140.

BLAUSTEIN, AR; BANCROFT, BA - Amphibian population declines: evolutionary considerations. **BioScience**. 57:5 (2007).

BLAUSTEIN, AR; WAKE, DB; SOUSA, WP - Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. **Conservation Biology**. (1994).

BLEM, C. R.; BLEM, L. B. - Tolerance of acidity in a Virginia population of the spotted salamander *Ambystoma maculatum* (Amphibia: Ambystomatidae). **Brimleyana**. 15 (1989) 37–45.

BOONE, D.Michelle; STACY, M.James - Interactions of an insecticide, herbicide and nastural stressors in amphibian community mesocosms. **Ecological Applications**. 13:3 (2003) 829–841.

BOONE, M. D.; BRIDGES, C. M.; ROTHERMEL, B. B. - Growth and development of larval green frogs (*Rana clamitans*) exposed to multiple doses of an insecticide. **Oecologia**. 129 (2001) 518–524.

BOONE, M. D.; SEMLITSCH, R. D. - Interactions of an insecticide with competition and pond drying in amphibian communities. **Ecological Applications**. 12 (2002) 43–52.

BRIDGES, C. M. - Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). **Archives of Environmental Contamination and Toxicology**. 39 (2000) 91–96.

BRIDGES, CM; SEMLITSCH, RD - Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. **Conservation Biology**. 14:5 (2000) 1490–1499.

BRIDGES, CM; SEMLITSCH, RD - Genetic variation in insecticide tolerance in a population of southern leopard frogs

(*Rana sphenocephala*): implications for amphibian conservation. **Copeia**. (2000).

BRITSON, C. A.; THRELKELD, S. T. - Abundance, metamorphosis, developmental, and behavioral abnormalities in *Hyla chrysoscelis* tadpoles following exposure to three agrochemicals and methyl mercury in outdoor mesocosms. **Bulletin of Environmental Contamination and Toxicology**. 61 (1998) 154–161.

BROWN, BE - Lead detoxification by a copper-tolerant isopod. **Nature**. 276, (1978) 388 - 390 doi:10.1038/276388a0.

BRÜHL, C.; SCHMIDT, T.; PIEPER, S.; ALSCHER, A.. - Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? **Scientific reports**. (2013) 1–4. doi: 10.1038/srep01135.

BUSKIRK, J. Van - A comparative test of the adaptive plasticity hypothesis: relationships between habitat and phenotype in anuran larvae. **The American Naturalist**. (2002).

BUSKIRK, Josh V. A. N.; RELYEA, Rick A.; ARBOR, A. - Selection for phenotypic plasticity in *Rana sylvatica* tadpoles. **Biological Journal of the Linnean Society**. 65 (1998) 301–328.

CALOW, P. - Physiological costs of combating chemical toxicants: ecological implications. **Comp. Biochemcal Physiology** 100:C (1991) 3–6.

CAREY, C; BRYANT, C - Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. **Environmental Health Perspectives**. 1995) 13–17.

CARR, L. W.; FAHRIG, L. - Impact of road traffic on two amphibian species of differing vagility. **Conservation Biology**. 15 (2001) 1071–1078.

CARVALHO, SB; BRITO, JC - Incorporating evolutionary processes into conservation planning using species distribution data: a case study with the western Mediterranean herpetofauna. **Diversity and Distributions**. (2011) 1–14. doi: 10.1111/j.1472-4642.2011.00752.x.

CASSEL, A.; WINDING, J.; NYLIN, S.; WIKLUND, C. - Effects of Population Size and Food Stress on Fitness-Related Characters in the Scarce Heath, a Rare Butterfly in Western Europe. **Conservation Biology**. 15:6 (2001) 1667–1673.

CBD -**Convention on Biological Diversity** (2013). Avalaible at WWW:<URL:http://www.cbd.int/>.

CHEN, Shao-Yu *et al.* - Extremely Low Genetic Diversity Indicating the Endangered Status of *Ranodon sibiricus* (Amphibia: Caudata) and Implications for Phylogeography. **PLoS ONE**. ISSN 1932-6203. 7:3 (2012) e33378. doi: 10.1371/journal.pone.0033378.

CHEN, T. H.; GROSS, J. A.; KARASOV, W. H. - Sublethal effects of lead on northern leopard frog (*Rana pipiens*) tadpoles. **Environ. Toxicol. Chem.** 25:2006) 1383–1389.

CHEN, T. H.; GROSS, J. A.; KARASOV, W. H. - Adverse effects of chronic copper exposure in larval northern leopard frogs (*Rana pipiens*). **Environ. Toxicol. Chem.** 26 (2007) 1470–1475.

CHEN, Te-Hao; GROSS, Jackson A; KARASOV, William H. - Chronic exposure to pentavalent arsenic of larval leopard frogs (*Rana pipiens*): bioaccumulation and reduced swimming performance. **Ecotoxicology**. ISSN 1573-3017. 18:5 (2009) 587–93. doi: 10.1007/s10646-009-0316-3.

CHI-SHIUN, W. *et al.* - Salinity acclimation enhances salinity tolerance in tadpoles living in brackish water through increased Na+, K+ - ATPase expression. **Journal of experimental biology.** 31A (2014) 57–64.

CLARK, D. R. Jr. CANTU, R.; COWMAN, D.F.; MAXSON, D.J. - Uptake of arsenic and metals by tadpoles at an

historically contaminated Texas site. **Ecotoxicology**. 7 (1998) 61–67.

CLARK, K. L. - Responses of the spotted salamander, *Ambystoma maculatum*, populations in central Ontario to habitat acidity. **Canadian Field Naturalist**. 100 (1986) 463-469.

COBBETT, Christopher; GOLDSBROUGH, Peter – Phytochelatins and Mettallothioneins: Roles in Heavy Metal Detoxification and Homeostasis. (2002). doi: 10.1146/annurev.arplant.53.100301.135154.

COLLINS, James P.; STORFER, Andrew - Global amphibian declines : sorting the hypotheses. **Diversity and Distributions**. 9 (2003) 89–98.

COOK, R. P. - Effects of acid precipitation on embryonic mortality of *Ambystoma* salamanders in the Connecticut Valley of Massachusetts. **Biological Conservation**. 27 (1983) 77–88.

CORN, P. S.; VERTUCCI, F. A. - Descriptive risk assessment of the effects of acidic deposition on Rocky Mountain amphibians. **Journal of Erpethology**. 26 (1992) 361–369.

COUSTEAU, C.; CHEVILLON, C.; FFRENCH-CONSTANT, R. H. - Resistance to xenobiotics and parasites: Can we count the cost? **Trends in Ecology & Evolution**. 15 (2000) 378–383.

COUTELLEC, Marie-Agnès; BARATA, Carlos - An introduction to evolutionary processes in ecotoxicology. **Ecotoxicology (London, England)**. . ISSN 1573-3017. 20:3 (2011) 493–6. doi: 10.1007/s10646-011-0637-x.

COYLE, P. - Metallothionein: The multipurpose protein. **Cellular and Molecular Life Sciences**. 59 (2002) 627–647.

DALE, J. M.; FREEDMAN, B.; KEREKES, J. - Experimental studies of the effects of acidity and associated water chemistry on amphibians. **Proceedings of the N. S. Inst. Sci.** 35 (1985) 35–54.

DARLING, E. S.; MCCLANAHAN, T. R.; COTE, I. - Life histories predict coral community disassembly under multiple stressors. **Global Change Biology**. 19 (2013) 1930–1940.

DARLING, Emily S.; MCCLANAHAN, Timothy R.; CÔTÉ, Isabelle M. - Combined effects of two stressors on Kenyan coral reefs are additive or antagonistic, not synergistic. **Conservation Letters**. . ISSN 1755263X. 3:2 (2010) 122–130. doi: 10.1111/j.1755-263X.2009.00089.x.

DAUM, J.; Davis, LEYLA, R.; BIGLER, L.; WOODHAMS, D. C. - Hybrid advantage in skin peptide immune defenses of water frogs (*Pelophylax esculentus*) at risk from emerging pathogens. **Infection, Genetics and Evolution**. . ISSN 1567-1348. 12:8 (2012) 1854–1864. doi: 10.1016/j.meegid.2012.07.024.

DAVID, Patrice - Heterozygosity–fitness correlations: new perspectives on old problems. **Heredity**. 80:November 1997 (1998) 531–537.

DAVIDSON, C.; SHAFFER, H. B.; JENNINGS, M. R. - Spatial tests of the pesticide drift, habitat destruction, UV-B and climate change hypotheses for California amphibian declines. **Conservation biology**. 16 (2002) 1588–1601.

DEWITT, T. J.; SIH, A.; WILSON, D. S. - Costs and limits of phenotypic plasticity. **Trends in ecology & evolution**. 13:2 (1998) 77–81. doi: http://dx.doi.org/10.1016/S0169-5347(97)01274-3.

DIANA, S. G.; RESETARIS, W.J.; SCHAEFFER, D.J.; BECKMEN, K.B.; BEASLEY, V.R. - Effects of atrazine on amphibian growth and survival in artificial aquatic communities. **Environ. Toxicol. Chem.** 19 (2000) 2961–2967.

DIECKMANN, U.; DOEBELI, M. - Adaptive dynamics of speciation: sexual populations. **Adaptive speciation**. (2004).

DINUDOM, Anuwat; VOYLES, Jamie; COOK, David I.; CAMPBELL, Craig R. - Frog skin epithelium: Electrolyte

transport and chytridiomycosis. **The International Journal of Biochemistry & Cell Biology**. ISSN 1878-5875. 44:3 (2012) 431–434. doi: 10.1016/j.biocel.2011.12.002.

DONG, Bingjun; CHE, Jing; DING, Li - Hypotheses of Pleistocene Population History Using Coalescent Simulations: Refugial Isolation and Secondary Contact in *Pseudepidalea raddei*. **Asian Journal of Herpetology**. 3:2 (2012) 103–113. doi: 10.3724/SP.J.1245.2012.00103.

DROST, C. A.; FELLERS, G. M. - Collapse of a regional frog fauna in the Yosemite area of the California Sierra Nevada, USA. **Conservation Biology**. 10 (1996) 414–425.

DUNSON, W. A.; CONNELL, J. - Specific inhibition of hatching in amphibian embryos by low pH. **Journal of Erpethology**. 3 (1982) 314–316.

DUNSON, W. A.; WYMAN, R. L.; CORBETT, E. S. - A symposium on amphibian declines and habitat acidification. **Journal of Herpetology**. 26 (1992) 349–352.

EARL, JE; SEMLITSCH, RD - Carryover effects in amphibians: Are characteristics of the larval habitat needed to predict juvenile survival? **Ecological Applications**. (2013).

FEDORENKOVA, A.; VONK, JA - Ranking ecological risks of multiple chemical stressors on amphibians. **Environmental Toxicology and Chemistry**. 31:6 (2012) 1416–1421. doi: 10.1002/etc.1831.

FERGUSON, D. E.; GILBERT, C. C. - Tolerances of three species of anuran amphibians to five chlorinated hydrocarbon insecticides. **J. Miss. Acad. Sci.** 13 (1967) 135–138.

FICETOLA, G. F.; PADOA-SCHIOPPA, E.; WANG, J.; GARNER, T. W. J.. - Polygyny, census and effective population size in the threatened frog, *Rana latastei*. **Animal Conservation**. ISSN 13679430. 13:2010) 82–89. doi: 10.1111/j.1469-1795.2009.00306.x.

FICETOLA, Gentile Francesco; GARNER, Trenton W. J.; DE BERNARDI, Fiorenza - Genetic diversity, but not hatching success, is jointly affected by postglacial colonization and isolation in the threatened frog, *Rana latastei*. **Molecular ecology**. ISSN 0962-1083. 16:9 (2007) 1787–97. doi: 10.1111/j.1365-294X.2006.03198.x.

FIORAMONTI, E. SEMLITSCHT, R.D.; FENT, K.; REYER, H. - Effects of triphenyltin and pH on the growth and development of *Rana lessonae* and *Rana esculenta* tadpoles. **Evironmental Toxicology and Chemistry**. 16:9 (1997) 1940–1947.

FISKER, Karina V.; SORENSEN, Jesper G; DAMGAARD, Christian; PEDERSEN, Knud Ladegaard; HOLMSTRUP, Martin - Genetic adaptation of earthworms to copper pollution: is adaptation associated with fitness costs in *Dendrobaena octaedra*? **Ecotoxicology (London, England)**. ISSN 1573-3017. 20:3 (2011) 563–573. doi: 10.1007/s10646-011-0610-8.

FOLT, C. L.; CHEN, C.Y.; MOORE, M.V.; BURNAFORD, J. - Synergism and antagonism among multiple stressors. **Limnology and Oceanography**. 4 (1999) 864–877.

FORBES, Valery E.; CALOW, Peter - Costs of Living with Contaminants: Implications for Assessing Low-Level Exposures. **BELLE Newsletter**. 4:3 (1996) 1–13.

FREDA, J. - The influence of acidic pond water on amphibians: A review. **Water Air Soil Pollution**. 20 (1986) 439–450.

FREDA, J. - The effects of aluminum and other metals on amphibians. **Environmental Pollution**. 71 (1991) 305–328.

FREDA, J.; DUNSON, W. A. - Effects of low pH and other chemical variables on the local distribution of amphibians. **Copeia**. (1986) 454–466.

GARCIA, T. S.; STACY, J.; SIH, A. - Larval salamander response to UV radiation and predation risk: color change and microhabitat use. **Ecological Applications**. 14 (2004) 1055–1064.

GHALAMBOR, C. K. *et al.* - Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. **Functional Ecology**. ISSN 0269-8463. 21:3 (2007) 394–407. doi: 10.1111/j.1365-2435.2007.01283.x.

GIBBONS, MM; MCCARTHY, TK - The reproductive output of frogs *Rana temporaria* (L.) with particular reference to body size and age. **Journal of Zoology**. (1986).

GIBBS, J. P.; WHITELEATHER, K. K.; SCHUELER, F. W. - Changes in frog and toad populations over 30 years in New york state. **Ecological Applications**. 15:4 (2005) 1148–1157.

GILLARDIN, V.; SILVESTRE, F.; DIEUO, M.; EDOUARD, D.; RAES, M.; THOME, J.; KESTEMONT, P. - Protein expression profiling in the african clawed frog *Xenopus laevis* tadpoles exposed to the polychlorinated biphenyl mixture Aroclor 1254s. **Molecular & Cellular Proteomics.** 8,4 (2009) 596–611.

GOMEZ-MESTRE, I.; TEJEDO, M. - Local adaptation of an anuran amphibian to osmotically stressful environments. **Evolution**. 57:8 (2003) 1889–1899.

GOMULKIEWICZ, R.; KIRKPATRICK, M. - Quantitative Genetics and the Evolution of Reaction Norms. **Evolution**. 46:2 (1992) 390–411.

GOYER, R. A.; CLARKSON, T. W. - Toxic effects of metals. Em KLAASSEN, C. D. (Ed.) - **Casarett and Doull's toxicology: the basic science of poisons.** [S.l.] : McGraw-Hill Professional, New York, NY, USA., 2001. p. 811–867.

GREEN, D. M. - The ecology of extinction: population fluctuation and decline in amphibians. **Biological Conservation**. . ISSN 00063207. 111:3 (2003) 331–343. doi: 10.1016/S0006-3207(02)00302-6.

HALL, R. J.; HENRY, P. F. P. - Assessing effects of pesticides on amphibians and reptiles: status and needs. **Herpetological Journal**. 2 (1992) 65–71.

HALVERSON, MA; SKELLY, DK; CACCONE, A. - Inbreeding linked to amphibian survival in the wild but not in the laboratory. **Journal of Heredity**. 97:5 (2006) 499–507. doi: 10.1093/jhered/esl019.

HANGARTNER, S. - The quantitative genetic basis of adaptive divergence in the moor frog (*Rana arvalis*) and its implications for gene flow. **Journal of Evolutionary Ecology** 25 (2012) 1587–1599. doi: 10.1111/j.1420-9101.2012.02546.x.

HANSSON, B.; WESTERBERG, L. - On the correlation between heterozygosity and fitness in natural populations. **Molecular Ecology**. (2002).

HATCH, A. C.; BLAUSTEIN, A. R. - Combined Effects of UV-B, Nitrate, and Low pH Reduce the Survival and Activity Level of Larval Cascades Frogs (*Rana cascadae*). **Arch. Environ. Contam. Toxicol.** 39 (2000) 494–499.

HATCH, A. C.; BLAUSTEIN, A. R. - Combined effects of UV-B radiation and nitrate fertilizer on larval amphibians. **Ecological Applications**. 13 (2003) 1083–1093.

HAYES, M. P.; PEARL, C. A.; ROMBOUGH, C. J. - *Rana aurora aurora* movement. **Herpetological Review**. 31 (2001)

35–36.

HAYES, T. B.; COLLINS, A.; LEE, M.; MENDOZA, M.; NORIEGA, N.; STUART, A.A.; VONK, A. - Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. **PNAS**. 99:8 (2002) 5476–5480.

HECNAR, S. J. - Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern Ontario. **Environ. Toxicol. Chem.** 14 (1995) 2131–2137.

HEDRICK, Philip W.; KALINOWSKI, Steven T. – Inbreeding and depression in conservatio biology. **Annual Review of Ecology and Systematics**. 31 (2000) 139–162.

HEDRICK, PW - Conservation genetics: where are we now? **Trends in Ecology & Evolution**. 16:11 (2001) 629–636.

HOBAN, Sean M.; HAUFFE, Heidi C.; PEREZ-ESPONA, Sílvia; ARNTZEN, Jan W.; BERTORELLE, Giorgio; BRYJA, Josef; FRITH, Katie; GAGGIOTTI, Oscar E.; GALBUSERA, Peter; GODOY, José; HOELZEL, A. RUS; Nichols, RICHARD A.; PRIMMER, CRAIG, R.; RUSSO, Isa-Rita; Segelbacher, Gernot; Siegismund, Hans R.; Sihvonen, Marjatta; Vernesi, Cristiano; VILÀ, Carles; BRUFORD, Michael W. - Bringing genetic diversity to the forefront of conservation policy and management. **Conservation Genetics Resources**. ISSN 1877-7252. 5:2 (2013) 593–598. doi: 10.1007/s12686-013-9859-y.

HOFFMANN, M.; HILTON-TAYLOR, C.; ANGULO, A. - The impact of conservation on the status of the world's vertebrates. **Sciencexpress**. (2010) 1–10.

HOPKINS, W. A.; CONGDON, J.; RAY, J. K. - Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. **Environ. Toxicol. Chem.** 29 (2000) 862–868.

HORNE, M. T.; DUNSON, W. A - The interactive effects of low pH, toxic metals, and DOC on a simulated temporary pond community. **Environmental Pollution**. . ISSN 0269-7491. 89:2 (1995) 155–61.

HORNE, M. T.; DUNSON, W. A. - Effects of low pH , metals , and water hardness on larval amphibians. **Arch. Environ. Contam. Toxicol.** 29 (1995) 500–505.

HOULAHAN, J. E.; FINDLAY, C.S.; SCHMIDT, B. R.; MEYER, A. H.; KUZMIN, S. L. - Quantitative evidence for global amphibian population declines. **Nature**. 404 (2000) 752–755.

HUEY, D. W.; BEITINGER, T. L. - Toxicity of nitrite of the Salamander *Ambystoma texanum*. **Bull. Environ. Contam. Toxicol.** 25 (1980) 909–912.

HUEY, R. B.; SLATKIN, M. - Cost and benefits of lizard thermoregulation. **The Quarterly Review of Biology**. 51 (1976) 363–384.

IRELAND, P. H. - Separate effects of acid-derived anions and cations on growth of larval salamanders of *Ambystoma maculatum*. **Copeia**. (1991) 132–137.

IUCN - **IUCN** [On line]. 2013. Available at WWW:<URL:http://www.iucn.org/>.

JAMES, S. M.; LITTLE, E. E.; SEMLITSCH, R. D. - The effect of soil composition and hydration on the bioavailability and toxicity of cadmium to hibernating juvenile American toads (*Bufo americanus*). **Environmental Pollution**. 132 (2004) 523–532.

JEHLE, R.; ARNTZEN, JW - Review: microsatellite markers in amphibian conservation genetics. **Herpetological**

Journal. 12 (2002) 1–9.

JUNG, R. E.; JAGOE, C. H. - Effects of low pH and aluminum on body size, swimming performance, and susceptibility to predation of green tree frog (*Hyla cinerea*) tadpoles. **Canadian Journal of Zoology**. 73 (1995) 2171–2183.

KARASOV, W. H. JUNG, R.E.; LANGENBERG, S.V.; BERGESON, T.L.E. - Field exposure of frog embryos and tadpoles along a pollution gradient in the Fox River and Green Bay ecosystem in Wisconsin, USA. **Environ. Toxicol. Chem.** 24 (2005) 942–953.

KELLER, LF; WALLER, DM - Inbreeding effects in wild populations. **Trends in Ecology & Evolution**. (2002).

KIESECKER, Joseph M.; BLAUSTEIN, Andrew R. - Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. **Proceedings of the National Academy of Sciences of the United States of America**. 92 (1995) 11049–11052.

KIESECKER, Joseph M.; BLAUSTEIN, Andrew R.; BELDEN, L. K. - Complex causes of amphibian population declines. **Nature**. 410 (2001) 681–684.

KNUTSON, M. G. SAUER, J. R.; OLSEN, D. A.; MOSSMAN, M. J.; HEMESATH, L. M.; LANNOO, M. J. - Effects of landscape composition and wetland fragmentation on frog and toad abundance and species richness in Iowa and Wisconsin, U.S.A. **Conservation biology**. 13 (1999) 1437–1446.

KOEHN, R. K.; BAYNE, B. L. - Towards a physiological and genetical understanding of the energetics of the stress response. **Biol. J. Linn. Soc.** 37 (1989) 151–171.

KUZMIN, S. - ***Pelophylax esculentus*.** [On line], 2009. Avaliable at :www.iucnredlist.org.

LAAN, R.; VERBOOM, B. - Effects of pool size and isolation on amphibian communities. **Conservation biology**. 54 (1990) 251–262.

LANGDON, CJ; PIEARCE, TG; BLACK, S; SEMPLE, KT - Resistance to arsenic-toxicity in a population of the earthworm *Lumbricus rubellus*. **Soil Biology**. (1999).

LANGDON, CJ; PIEARCE, TG - Resistance to copper toxicity in populations of the earthworms *Lumbricus rubellus* and *Dendrodrilus rubidus* from contaminated mine wastes. **Environ Toxicol Chem.** 20:10 (2001), 2336-41.

LANGHELLE, A.; LINDELL, M. J.; NYSTROM, P. - Effects of ultraviolet radiation on amphibian embryonic and larval development. **Journal of Herpetology**. 33 (1999) 449–456.

LAUDET, V. - The Origins and Evolution of Vertebrate Metamorphosis. **Current Biology**. (2011).

LAURILA, A.; KARTTUNEN, S.; MERILÄ, J. - Adaptive phenotypic plasticity and genetics of larval life histories in two *Rana temporaria* populations. **Evolution**. 56:3 (2002) 617–627.

LESBARRÈRES, David; PRIMMER, Craig R; LAURILA, Anssi; MERILA, Juha - Environmental and population dependency of genetic variability-fitness correlations in *Rana temporaria*. **Molecular ecology**. ISSN 0962-1083. 14:1 (2005) 311–323. doi: 10.1111/j.1365-294X.2004.02394.x.

LICHT, L. E. - Shedding light on ultraviolet radiation and amphibian embryos. **Bioscience**. 53 (2003) 551–561.

LINDER, G.; GRILLITSCH, B. - Ecotoxicology of metals. **Ecotoxicology of Amphibians and Reptiles. SETAC**. (2000).

LITVINCHUK, Spartak N.; BIOLLAY, Sébastien; BERONEAU, Matthieu; BORZEE, Amaël; OLGIESKA, Maria;

DUFERSNES, Christophe; LYMBERAKYS, Petros; GHALI, Karim; PERRIN, Nicolas; STOCK, Matthias - Cryptic diversity among Western Palearctic tree frogs: Postglacial range expansion, range limits, and secondary contacts of three European tree frog lineages (*Hyla arborea* group). **Molecular Phylogenetics and Evolution**. ISSN 1095-9513. 65:1 (2012) 1–9. doi: 10.1016/j.ympev.2012.05.014.

LIU, Y. S. - Inheritance of acquired characters in animals: A historical overview, further evidence and mechanistic explanations. **Italian Journal of Zoology**. ISSN 1125-0003. 78:4 (2011) 410–417. doi: 10.1080/11250003.2011.562554.

LOPES, Isabel; MARTINS, Nelson; BAIRD, Donald J; RIBEIRO, Rui. - Genetic Erosion and population resilience in *Daphnia Longispina* O.F. Muller under simulated predation and metal pressures. **Environmental Toxicology and Chemistry**. 28:9 (2009) 1912–1919.

LOPES, Isabel; BAIRD, Donald J.; RIBEIRO, Rui - Genetically determined resistance to lethal levels of copper by *Daphnia longispina*: association with sublethal response and multiple/coresistance. **Environmental toxicology and chemistry / SETAC**. 24:6 (2005) 1414–1419.

LUQUET, E. LENA, J.; DAVID, P.; JOLY, P.; LENGAGNE, T.; PERRIN, N.; PLENET, S. - Consequences of genetic erosion on fitness and phenotypic plasticity in European tree frog populations (*Hyla arborea*). **Journal of evolutionary biology**. ISSN 1420-9101. 24:1 (2011) 99–110. doi: 10.1111/j.1420-9101.2010.02138.x.

LUQUET, E; DAVID, P; LENA, J; JOLY, P; KONECNY, L; DUFRESNES, C; PERRIN, N; PLENET, S - Heterozygosity-fitness correlations among wild populations of European tree frog (*Hyla arborea*) detect fixation load. **Molecular ecology**. ISSN 1365-294X. 20:9 (2011) 1877–1887. doi: 10.1111/j.1365-294X.2011.05061.x.

LUQUET, E.; GARNER, T.W.J.; LENA, J.; BRUEL, C. - Genetic erosion in wild populations makes resistance to a pathogen more costly. **International journal of organic evolution**. (2012).

LUQUET, E.; LENA, J.; DAVID, P.; PRUNIER, J.; JOLY, P.; LENGAGNE, T.; PERRINAND, N.; PLENET, S. - Within- and among-population impact of genetic erosion on adult fitness-related traits in the European tree frog Hyla arborea. **Heredity**. 110 (2013) 347–354. doi: 10.1038/hdy.2012.110.

MACÌAS, G.; MARCO, A.; BLAUSTEIN, A. R. - Combined exposure to ambient UVB radiation and nitrite negatively affects survival of amphibian early life stages. **Science of the Total Environment**. 385 (2007) 55–65.

MACKIE, JA; LEVINTON, JS - Loss of evolutionary resistance by the oligochaete *Limnodrilus hoffmeisteri* to a toxic substance—cost or gene flow? **Evolution**. 1964 (2010). doi: 10.1111/j.1558-5646.2009.00806.x.

MAIORANO, Luigi *et al.* - Threats from Climate Change to Terrestrial Vertebrate Hotspots in Europe. **PloS one**. 8:9 (2013) 1–14. doi: 10.1371/journal.pone.0074989.

MARCO, A.; BLAUSTEIN, A. R. - The effects of nitrite on behavior and metamorphosis in cascades frogs (*Rana cascadae*). **Environ. Toxicol. Chem.** 18 (1998) 946–949.

MARCO, A.; QUILCHANO, C.; BLAUSTEIN, A. - Sensitivity to nitrate and nitrite in pond-breeding amphibians from the pacific northwest, USA. **Environmental Toxicology and Chemistry**. 18:12 (1999) 2836–2839.

MAXWELL, P. S. PITT, K.A.; BURFEIND, D.D.; OLDS, A.D.; BABCOCOK, R.C.; CONNOLLY, R.M. - Phenotypic plasticity promotes persistence following severe events: physiological and morphological responses of seagrass to flooding. **Journal of Ecology**. 110 (2014) 54–64.

MAZANTI, L.; SPARLING, D.W.; RICE, C.; BIALEK, K.; STEVENSON, C.; TEELS, B. - Synergistic effects of a

combined exposure to herbicides and an insecticide in Hyla versicolor. Em LINDER, G. I. - Multiple Stressor Effects in Relation to Declining Amphibian Populations. [S.l.] : (Eds.) **American Society for Testing and Materials** (2003).

MCKENZIE, VJ; PETERSON, AC - Pathogen pollution and the emergence of a deadly amphibian pathogen. **Molecular ecology**. (2012) 5151–5154.

MEDINA, MH; CORREA, JA; BARATA, Carlos - Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. **Chemosphere**. 67 (2007) 2105–2114. doi: 10.1016/j.chemosphere.2006.12.024.

MERILA, J.; LAURILA, A.; LAUGEN, T.A.; RASANEN, K.; PAHKALA, M. - Plasticity in size and age at metamorphosis in *Rana temporaria* - comparison of high and low latitude populations. **Ecography**. 23 (2000) 457–465.

MERILA, J.; LAURILA, A.; LAUGEN, T.A.; RASANEN, K.; PAHKALA, M. - Adaptive phenotypic plasticity in timing of metamorphosis in the common frog *Rana temporaria*. **Ecoscience**. 7 (2000) 18–24.

MERILÄ, J.; SHELDON, B.C. - Genetic architecture of fitness and nonfitness traits: empirical patterns and development of ideas. **Heredity**. (1999).

MIAUD, Claude; GUYÉTANT, R.; ELMBERG, Johan - Variations in life-history traits in the common frog *Rana temporaria* (Amphibia: Anura): a literature review and new data from the French Alps. **Journal of Zoology**. (1999).

MUELLER, Casey A; AUGUSTINE, Starrlight; KOOIJMAN, Sebastiaan A L M; KEARNEY, Michael R; SEYMUR, Roger S - Comparative Biochemistry and Physiology , Part A: The trade-off between maturation and growth during accelerated development in frogs. **Comparative Biochemistry and Physiology**. ISSN 1095-6433. 163:1 (2012) 95–102. doi: 10.1016/j.cbpa.2012.05.190.

NATARAJ, Makkimane B.; KRISHNAMURTHY, Sannanegunda V - Effects of combinations of malathion and cypermethrin on survivability and time of metamorphosis of tadpoles of Indian cricket frog (*Fejervarya limnocharis*). **Journal of environmental science and health. Part. B, Pesticides, food contaminants, and agricultural wastes**. ISSN 1532-4109. 47:2 (2012) 67–73. doi: 10.1080/03601234.2012.611428.

NEWMAN, RA - Adaptive plasticity in development of Scaphiopus couchii tadpoles in desert ponds. **Evolution**. 42 (1988) 774–783.

NEWMAN, RA - Adaptive plasticity in amphibian metamorphosis. **Bioscience**. (1992).

NICIEZA, AG - Interacting effects of predation risk and food availability on larval anuran behaviour and development. **Oecologia**. (2000) 497–505.

NOWAK, Carsten; VOGT, Christian; PFENNINGER, Markus - Rapid genetic erosion in pollutant-exposed experimental chironomid populations. **Environmental Pollution**. 157:2009) 881–886. doi: 10.1016/j.envpol.2008.11.005.

NUÑEZ, José J; WOOD, Nicole K; RABANAL, Felipe E; FONTANELLA, Frank M; SITES, Jack W - Amphibian phylogeography in the Antipodes: Refugia and postglacial colonization explain mitochondrial haplotype distribution in the Patagonian frog *Eupsophus calcaratus* (Cycloramphidae). **Molecular phylogenetics and evolution**. ISSN 1095-9513. 58:2 (2011) 343–52. doi: 10.1016/j.ympev.2010.11.026.

O'BRIEN, John; ALLENTOFT, Morten E. - Global Amphibian Declines, Loss of Genetic Diversity and Fitness: A Review. **Diversity**. ISSN 1424-2818. 2:1 (2010) 47–71. doi: 10.3390/d2010047.

ORTIZ, M. E. MARCO, A.; SAIZ, N.I.; LIZANA, M. - Impact of ammonium nitrate on growth and survival of six European amphibians. **Arch. Environ. Contam. Toxicol.** 47 (2004) 234–239.

ORTIZ-SANTALIESTRA, M. E.; MARCO, A.; LIZANA, M. - Sensitivity and behavior of the iberian newt, *Triturus boscai*, under terrestrial exposure to ammonium nitrate. **Bull. Environ. Contam. Toxicol.** 75 (2005) 662–669.

ORTIZ-SANTALIESTRA, Manuel E.; MARCO, Adolfo; FERNANDEZ, María José; LIZANA, Miguel - Influence of developmental stage on sensitivity to ammonium nitrate of aquatic stages of amphibians. **Environmental toxicology and chemistry / SETAC**. ISSN 0730-7268. 25:1 (2006) 105–11.

ORTIZ-SANTALIESTRA, Manuel E.; MARCO, Adolfo; FERNANDEZ, María José; LIZANA, Miguel - Effects of ammonium nitrate exposure and water acidification on the dwarf newt: The protective effect of oviposition behaviour on embryonic survival. **Aquatic toxicology (Amsterdam, Netherlands)**. ISSN 0166-445X. 85:4 (2007) 251–7. doi: 10.1016/j.aquatox.2007.09.008.

ORTIZ-SANTALIESTRA, Manuel E.; FERNANDEZ, María José; LIZANA, Miguel; MARCO, Adolfo - Adaptation to osmotic stress provides protection against ammonium nitrate in *Pelophylax perezi* embryos. **Environmental pollution (Barking, Essex : 1987)**. ISSN 1873-6424. 158:3 (2010) 934–40. doi: 10.1016/j.envpol.2009.09.011.

OWE, G.Eorge E. H.; ILLIS, R. I. C. K. G.; OWBRAY, R.Odney C. M. - Effect of chemical synergy and larval stage on the toxicity of atrazine and alachlor to amphibian larvae. **Environmental Toxicology and Chemistry**. 17:3 (1998) 519–525.

OZA, Anuja U.; LOVETT, Katharine E; WILLIAMS, Stephen E; MORITZ, Craig - Molecular Phylogenetics and Evolution Recent speciation and limited phylogeographic structure in Mixophyes frogs from the Australian Wet Tropics. **Molecular Phylogenetics and Evolution**. ISSN 1055-7903. 62:1 (2012) 407–413. doi: 10.1016/j.ympev.2011.10.010.

PAHKALA, M.; LAURILA, A.; MERILA, J. - Ambient ultraviolet-B radiation reduces hatchling size in the common frog *Rana temporaria*. **Ecography**. 23:2000) 531–538.

PAHKALA, Maarit; LAURILA, Anssi; JOHANSON, U. L. F. - Lethal and sublethal effects of UV-B/pH synergism on common frog embyros. **Conservation Biology**. 16:4 (2002) 1063–1073.

PEARMAN, P. B.; GARNER, T. W. J. - Susceptibility of Italian agile frog populations to an emerging Ranavirus parallels population genetic diversity. **Ecology Letters**. 8 (2005) 401–408.

PECHMAN, J. H. K. H.; WILBUR, M. - Putting declining amphibian populations into perspective: Natural fluctuations and human impacts. **Herpetologica**. 50 (1994) 65–84.

PIERCE, B. A. - Acid tolerences in amphibians. **Bioscience**. 35:1985) 239–243.

PIERCE, BA.; SIKAND, N. - Variation in acid tolerance of Connecticut wood frogs: genetic and maternal effects. **Canadian Journal of Zoology**. 63 (1985) 1647–1651.

PIGLIUCCI, M.; SCHLICHTING, C. D. - Phenotypic evolution: a reaction norm perspective. (1997) Sinauer As ed., MA. 387 pp.

POLCÁK, D.; GVOZDÍK, L. - Should I stay or should I go? The influence of temperature and sex on predator-induced responses in newts. **Animal Behaviour**. 89 (2014) 79–84.

POUGH, F. H. - Acid precipitation and embryonic mortality of spotted salamanders, *Ambystoma maculatum*. **Science**. 192 (1976) 68–72.

POUGH, F. H.; KAMEL, S. - Post-metamorphic change in activity metabolism of anurans in relation to life history. **Oecologia**. 65 (1984) 138–144.

POUNDS, J.Alan *et al.* - Widespread amphibian extinctions from epidemic disease driven by global warming. **Nature**. . ISSN 1476-4687. 439:7073 (2006) 161–7. doi: 10.1038/nature04246.

PYRON, R.Alexander; WIENS, John J. - A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. **Molecular phylogenetics and evolution**. ISSN 1095-9513. 61:2 (2011) 543–83. doi: 10.1016/j.ympev.2011.06.012.

RADWAN, J.; DEMIASZKIEWICZ, A.W.; KOWALCZYK, R.; LACHOWICZ, J.; KAWALKO, A.; WOJCIK, J.M. - An evaluation of two potential risk factors, MHC diversity and host density, for infection by an invasive nematode Ashworthius sidemi in endangered European bison. **Biological Conservation**. 143 (2010) 2049–2053.

RASANEN, K.; LAURILA, A.; MERILA, J. - Geographic variation in acid stress tolerance of the moor frog, *Rana arvalis* I. Local adaptation. **Evolution**. 57 (2003) 352–362.

RÄSÄNEN, K.; LAURILA, A.; MERILÄ, J. - Carry-over effects of embryonic acid conditions on development and growth of *Rana temporaria* tadpoles. **Freshwater biology**. (2002) 19–30.

RÄSÄNEN, K.; LAURILA, A.; MERILÄ, J. - Maternal investment in egg size: environment-and population-specific effects on offspring performance. **Oecologia**. (2005) 546–553. doi: 10.1007/s00442-004-1762-5.

RAWSON, P. D.; HILBISH, T. J. - Genotype-environment interaction for juvenile growth in the hard clam *Mercenaria mercenaria* (L.). **Evolution**. 45 (1991) 1924–1935.

REED, D. H.; FRANKHAM, R. - Correlation between fitness and genetic diversity. **Conservation Biology**. 17:1 (2003) 230–237.

RELYEA, Rick A; DIECKS, Nicole - An unforeseen chain of events: lethal effects of pesticides on frogs at sublethal concentrations. **Ecological Applications**. ISSN 1051-0761. 18:7 (2008) 1728–42.

RIBEIRO, Rui; LOPES, Isabel - Contaminant driven genetic erosion and associated hypotheses on alleles loss, reduced population growth rate and increased susceptibility to future stressors: an essay. **Ecotoxicology (London, England)**. ISSN 1573-3017. 22:5 (2013) 889–99. doi: 10.1007/s10646-013-1070-0.

ROUSE, J. D.; BISHOP, C. A.; SRUGER, J. - Nitrogen pollution: an assessment of its threat to amphibian survival. **Environ. Health Perspect.** 107 (1999) 799–803.

ROWE, C. L.; HOPKINS, W. A.; COFFMAN, V. R. - Failed recruitment of southern toads (*Bufo terrestris*) in a trace element-contaminated breeding habitat: direct and indirect effects that may lead to a local population sink. **Arch. Environ. Contam. Toxicol.** 40 (2001) 399–405.

ROWE, C. L.; KINNEY, O. M.; CONGDON, J. D. - Oral deformities in tadpoles of the bullfrog (*Rana catesbeiana*) caused by conditions in a polluted habitat. **Copeia**. (1998) 244–246.

ROWE, C. L.; SADINSKI, W. J.; DUNSON, W. A. - Effects of acute and chronic acidification on three larval amphibians that breed in temporary ponds. **Arch. Environ. Contam. Toxicol.** 23 (1992) 339–350.

ROWE, C. R.; *Kinney, O.M.; Nagle, R.D.; Congdon, J.D.* - Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. **Physiological Zoology**. 71 (1998) 27–35.

ROWE, CL; FREDA, J. - Effects of acidification on amphibians at multiple levels of biological organization. **Ecotoxicology of amphibians and reptiles. SETAC**. (2000).

ROWE, G.; BEEBEE, TJC - Population on the verge of a mutational meltdown? Fitness costs of genetic load for an amphibian in the wild. **Evolution**. 57:1 (2003) 177–181.

SALA, OE; CHAPIN, FS; ARMESTO, JJ - Global biodiversity scenarios for the year 2100. **Science**. 287:March (2000) 1770–1774.

SANTOS, Bàrbara; RIBEIRO, Rui; DOMINGUES, Ines; PEREIRA, Ruth; SOARES, Amadeu M.V.M.; LOPES, Isabel. – Salinity and copper interactive effects on Perez's frog, *Pelophylax perezi*. **Environmental Toxicology and Chemistry**. 32:8 (2013) 1864–1872. doi: 10.1002/etc.2257.

SATO, Chloe F.; WOOD, Jeff T.; SCHRODER, Mellesa; GREEN, Ken; OSBORNE, William S.; MICHAEL, Damian R.; LYINDENMAYER, David B. - An experiment to test key hypotheses of the drivers of reptile distribution in subalpine ski resorts. **Journal of Applied Ecology**. ISSN 00218901. (2013) n/a-n/a. doi: 10.1111/1365-2664.12168.

SAVAGE, AE; ZAMUDIO, KR - MHC genotypes associate with resistance to a frog-killing fungus. **Proceedings of the National Acadmy of Science USA**. (2011) 1–6. doi: 10.1073/pnas.1106893108.

SCHEINER, S. M. - Genetics and evolution of phenotypic plasticity. **Annual Review of Ecology and Systematics**. 24 (1993) 35–68.

SCHEINER, S. M. - Plasticity as a selectable trait: reply to Via. **American Naturalist**. 142 (1993) 372–374.

SCHEINER, S. M.; LYMAN, R. F. - The genetics of phenotypic plasticity. II. Response to selection. **Journal of evolutionary biology**. 4 (1991) 23–50.

SCHMELLER, DS; SCHREGEL, Julia; VEITH, Michael - The importance of heterozygosity in a frog's life. **Naturwissenschaften**. (2007) 360–366. doi: 10.1007/s00114-006-0205-z.

SCHULTE, Patricia M. - What is environmental stress? Insights from fish living in a variable environment. **The Journal of experimental biology**. ISSN 1477-9145. 217:Pt 1 (2014) 23–34. doi: 10.1242/jeb.089722.

SCHUYTEMA, G. S.; NEBEKER, A. - Comparative effects of ammonium and nitrate compounds on Pacific treefrog and African clawed frog embryos. **Arch. Environ. Contam. Toxicol.** 36 (1999) 200–206.

SCHUYTEMA, G. S.; NEBEKER, A. V. - Effects of ammonium nitrate, sodium nitrate, and urea on red-legged frogs, Pacific treefrogs, and African clawed frogs. **Bull. Environ. Contam. Toxicol.** 63 (1999) 357–364.

SCOTT, D. E. - Effect of larval density on life history traits in Ambystoma opacum. **Ecology**. 75:1994) 1383–1396.

SEMLITSCH, R. D.; SCOTT, D.E.; PECHMANN, J.H.K.; GIBBONS, J.W. - Structure and dynamics of an amphibian community: evidence 184 from a 16-year study of a natural pond. Em CODY, M.L., SMALLWOOD (EDS) (Ed.) - **Long-term studies of vertebrate communities.** [S.l.] : Academic Press, San Diego., 1996. p. 217–248.

SEMLITSCH, R. D.; BRIDGES, C. M.; WELCH, A. M. - Genetic variation and a fitness tradeoff in the tolerance of gray treefrog (*Hyla versicolor*) tadpoles to the insecticide carbaryl. **Oecologia**. ISSN 0029-8549. 125:2 (2000) 179–185. doi: 10.1007/s004420000443.

SEMLITSCH, R. D.; SCOTT, D. E.; PECHMANN, J. H. K. - Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. **Ecology**. 69 (1988) 184–192.

SHAW, A. J. - The evolution of heavy metal tolerance in plants: adaptations, limits, and costs. Em FORBES, V. E. (Ed.) - **Genetics and ecotoxicology.** [S.l.] : Taylor and Francis, Philadelphia., 1999. p. 9–30.

SLOCUM, M. G.; MENDELSSOHN, I. A. - Effects of three stressors on vegetation in an oligohaline marsh. **Freshwater Biology**. 53 (2008) 1783–1796.

SMITH, D. C. - Adult recruitment in chorus frogs: effects of size and date at metamorphosis. **Ecology**. 68 (1987) 344–350.

SMITH, M.Alex; GREEN, David M. - Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? **Ecography**. 28:1 (2005) 110–128.

SNODGRASS, J. W.; HOPKINS, W.A.; BROUGHTON, J.; GWINN, D.; BAIONNO, J.A.; BURGER, J. - Species-specific responses of developing anurans to coal combustion wastes. **Aquatic Toxicology**. 66 (2004) 171–182.

SPARLING, DW; LINDER, G; BISHOP, CA; KREST, SK - Ecotoxicology of amphibians and reptiles. (2010). ISBN 9781420064162 - 944 Pages - CRC Press.

SPIELMAN, D; BROOK, B W; BRISCOE, D; FRANKHAM, R - Does inbreeding and loss of genetic diversity decrease disease resistance? **Conservation Genetics**. 5:4 (2004) 439–448.

SPURGEON, DJ; HOPKIN, SP - The development of genetically inherited resistance to zinc in laboratory-selected generations of the earthworm *Eisenia fetida*. **Environmental Pollution**. (2000).

STEBBINS, R. C.; COHEN, N. W. - A Natural History of Amphibians. Princeton ed. Princeton : [s.n.] (1997).

STEVENSON, LA; ALFORD, RA; BELL, SC; ROZNIK, EA - Variation in thermal performance of a widespread pathogen, the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. **PloSone**. 8:9 (2013) 1–14. doi: 10.1371/journal.pone.0073830.

STORFER, Andrew - Amphibian declines : future directions. **Diversity and Distributions**. 9 (2003) 151–163.

STRAALEN, NM VAN; JANSSENS, TKS; ROELOFS, Dick - Micro-evolution of toxicant tolerance: from single genes to the genome's tangled bank. **Ecotoxicology**. (2011) 574–579. doi: 10.1007/s10646-011-0631-3.

STRAALEN N.M., VAN; TIMMERMANS M.J.T.N. - Genetic variation in toxicants-stressed populations: An evaluation of the «Genetic Erosion» Hypothesis. (2002).

STUART, SN; CHANSON, JS; COX, NA - Status and trends of amphibian declines and extinctions worldwide. **Science**. 306 (2004) 1783–1786.

SULTAN, S. E.; SPENCER, H. G. - Metapopulation structure favors plasticity over local adaptation. **American Naturalist**. 160 (2002) 271–283.

TEACHER, A G. F.; GARNER, T. W. J.; NICHOLS, R. A - European phylogeography of the common frog (*Rana temporaria*): routes of postglacial colonization into the British Isles, and evidence for an Irish glacial refugium. **Heredity**. ISSN 1365-2540. 102:5 (2009) 490–6. doi: 10.1038/hdy.2008.133.

THRELKELD, S. T.; BRITSON, C. A. - Interactive effects of anthropogenic, environmental, and biotic stressors on multiple endpoints in *Hyla chrysoscelis*. **Journal of the Iowa Academy of Science**. 107 (2000) 61–66.

TINGLEY, Reid; DUBEY, Sylvain - Disparity in the timing of vertebrate diversification events between the northern and southern hemispheres. **BMC Evolutionary Biology**. ISSN 1471-2148. 12:1 (2012) 244. doi: 10.1186/1471-2148-12-244.

TRAVIS, J. - **Ecological genetics of life-history traits: variation and its evolutionary significance.** L. A. Real ed. (1994).

TUNNER, H. G. - Locomotion behavior in water frogs from Neusiedlersee. **Proceedings of the Sixth Ordinary General Meeting of the Societas Europaea Herpetologica.** Budapest : Hungarian National Museum (1992).

UITREGT, V. O. VAN; WILSON, R. S.; FRANKLIN, C. E. - Cooler temperatures increase sensitivity to ultraviolet B radiation in embryos and larvae of the frog *Limnodynastes peronii*. **Global Change Biology**. 13 (2007) 1114–1121.

VATNICK, I.; BRODKIN, M.A.; SIMON, M.; GRANT, B.W.; CONTE, C.R.; GLEAVE, M.; MYERS, R.; SADOFF, M.M. - The effects of exposure to mild acidic conditions on adult frogs (*Rana pipiens* and *Rana clamitans*): mortality rates and pH preferences. **Journal of Erpethology**. 33 (1999) 370–374.

VEITH, Michael; BAUMGART, Andrea; DUBOIS, Alain; OHLER, Annemarie; GALAN, Pedro; VIEITES, David R; NIETO-ROMAN, Sandra; VENCES, Miguel - Discordant patterns of nuclear and mitochondrial introgression in Iberian populations of the European common frog (*Rana temporaria*). **The Journal of heredity**. ISSN 1465-7333. 103:2 (2012) 240–9. doi: 10.1093/jhered/esr136.

VENCES, Miguel; HAUSWALDT, J Susanne; STEINFARTZ, Sebastian; RUPP, Oliver; GOEASMANN, Alexander; KUNZEL, Sven; vIEITES, David R; NIETO-ROMAN, Sandra; HAAS, Sabrina; LAUGHSCH, Clara; GEHARA, Marcelo; BRUCHMANN, Sebastian; PABIJAN, Maciej; LUDEWING, Ann-Kathrin; RUDERT, Dirk; ANGELINI, Claudio; BORKIN, Leo J; CROCHET, Pierre-André; CROTTINI, Angelica; DUBOIS, Alain; FICETOLA, Gentile Francesco; GALAN, Pedro; GENIEZ, Philippe; HACHTEL, Monika; JOVANOVIC, Olga; LYMBERAKIS, Petros; OHLER, Annemarie; SMIRNOV, Nazar - Radically different phylogeographies and patterns of genetic variation in two European brown frogs, genus *Rana*. **Molecular phylogenetics and evolution**. ISSN 1095-9513. 68:3 (2013) 657–70. doi: 10.1016/j.ympev.2013.04.014.

VERTUCCI, F. A.; CORN, P. S. - Evaluation of episodic acidification and amphibian declines in the Rocky Mountains. **Ecological Applications**. 6 (1996) 449–457.

VIA, S.; LANDE, R. - Genotype-environment interaction and the evolution of phenotypic plasticity. **Evolution**. 39:3 (1985) 502–522.

VIA, Sara - Adaptive phenotypic plasticity: target or by-product of selection in a variable environment? **American Naturalist**. 142 (1993) 352–365.

VIA, Sara; GOMULKIEWICZ, R.; JONG, G. De - Adaptive phenotypic plasticity: consensus and controversy. **Trends in Ecology & Evolution**. 5 (1995).

VINEBROOKE, R. D.; COTTINGHAM, K.L.; NORBERG, J.; SCHEFFER, M.; DODSON, S.I.; MABERLY, S.C.; SOMMER, U. - Impacts of multiple stressors on biodiversity and ecosystem functioning: the role of species co-tolerance. **Oikos**. 104 (2004) 451–457.

WAH CHU, K.; CHOW, K. L. - Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. **Aquatic Toxicology**. 61 (2002) 53–64.

WAKE, D. B. - Declining amphibian populations. **Science**. 253 (1991) 860.

WAKE, DB; VREDENBURG, VT - Are we in the midst of the sixth mass extinction? A view from the world of amphibians. **Proceedings of the National Academy of Sciences of the United States of America**. 105 (2008) 11466–11473.

WARNER, S. C.; DUNSON, W. A. - The Effect of Low pH on Amphibians Breeding in Temporary Ponds in North

Florida. **Florida Game and Fresh Water Fish Commission Final Report**, Tallahassee, Florida, USA. 87pp. (1991).

WATT, P. J.; JARVIS, P. - Survival analysis in palmate newts exposed to ammonium nitrate agricultural fertilizer. **Ecotoxicology**. 6 (1997) 355–362.

WATT, P. J.; OLDHAM, R. S. - The effect of ammonium nitrate on the feeding and development of the smooth newt, *Triturus vulgaris* (L.), and on the behaviour of its food source, Daphnia. **Freshwater Biology**. 33 (1995) 319–324.

WEITERE, Markus; TAUTZ, Diethard; NEUMANN, Dietrich; STEINFARTZ, Sebastian - Adaptive divergence vs. environmental plasticity: tracing local genetic adaptation of metamorphosis traits in salamanders. **Molecular ecology**. ISSN 0962-1083. 13:6 (2004) 1665–77. doi: 10.1111/j.1365-294X.2004.02155.x.

WHITEMAN, H. H.; HOWARD, R. D.; WHITTEN, K. A. - Effects of pH on embryo tolerance and adult behavior in the tiger salamander, *Ambystoma tigrinum tigrinum*. **Canadian Journal of Zoology**. 73 (1995) 1529–1537.

WILBUR, H. M. - Complex life cycles. **Annual Review of Ecological Systems**. 11 (1980) 67–93.

WILBUR, H. M.; COLLINS, J. P. - Ecological aspects of amphibian metamorphosis. **Science**. 182:1973) 1305–1314.

WILLI, Y.; BUSKIRK, J. Van; HOFFMANN, AA - Limits to the adaptive potential of small populations. **Annual Review of Ecology, Evolution, Systematics**. 37 (2006) 433–458.

WYMAN, R. L.; HAWKSLEY-LESCAULT, D. S. - Soil acidity affects distribution, behavior, and physiology of the salamander *Plethodon cinereus*. **Ecology**. 68 (1987) 1819–1827.

XU, Q.; OLDHAM, R. S. - Lethal and sublethal effects of nitrogen fertiliser ammonium nitrate on common toad (*Bufo bufo*) tadpoles. **Arch. Environ. Contam. Toxicol.** 32 (1997) 298–303.

ZAGA, A. - Photoenhanced toxicity of a carbamate insecticide to early life stage anuran amphibians. **Environmental Toxicology and Chemistry**. 17 (2008) 2543–2553.

ZEISSET, I.; BEEBEE, T. J. C. - Amphibian phylogeography: a model for understanding historical aspects of species distributions. **Heredity**. ISSN 1365-2540. 101:2 (2008) 109–19. doi: 10.1038/hdy.2008.30

CHAPTER 3.

Inheritance of tolerance to metals: Among and within amphibian egg masses variability may unveil trait dominance.

Inheritance of tolerance to metals:
Among and within amphibian egg masses variability may unveil trait dominance.

Abstract:

Chemical contamination may cause genetic erosion in natural populations (decrease of genetic diversity) by wiping out the most sensitive genotypes. This is of particular concern if the loss of genetic variability is irreversible due to the contaminant-driven elimination of alleles, which theoretically could happen if tolerance is a recessive or incompletely dominant trait – the recessive tolerance inheritance (working-) hypothesis put forward by R. Ribeiro and I. Lopes in 2013. Accordingly, this work aimed at investigating the inheritance, in natural populations of the Perez's frog (*Pelophylax perezi*), of the tolerance to a lethal level of a metals-rich acid mine drainage sample. Twenty-one egg masses, at Gosner's stages 8 to 10, were collected from a population inhabiting a reference pond. Time to death was registered for each egg. For each egg mass, the median lethal time (LT_{50}) and respective quartiles (LT_{25} and LT_{75}) were computed. The within egg mass variability in time to death responses was evaluated through its relative spread: the difference between the lower and upper quartiles relatively to the median: $(LT_{75}-LT_{25})/LT_{50}$. Our results unveil how genetic mechanism of tolerance to lethal levels of acid mine drainage might probably be determined by incomplete dominance, preliminarily supporting the hypothesis.

Keywords: Inheritance, recessive trait, tolerance, incomplete dominance, acid mine drainage, genetic erosion, Perez's frog, *Pelophylax perezi*.

Introduction

The designation "genetic erosion" and concerns about its occurrence are now explicit at the political world-level through the United Nations decision of adopting the Strategic Plan for Biodiversity 2011-2020 (CBD 2010). The mandatory implementation of "strategies […] for minimizing genetic erosion" targets explicitly the species-level (not the population-level) and only some species: "cultivated plants and farmed and domesticated animals and of wild relatives, including other socio-economically as well as culturally valuable species" (from the Aichi Biodiversity Target 13, Strategic goal C: Improve the status of biodiversity by safeguarding ecosystems, species and genetic diversity). However, the

conservation of threatened species, which is also aimed at (Target 12), implies halting the impoverishment of the genetic diversity of local populations. Alongside other menaces, contaminant-driven genetic erosion has been increasingly reported (Van Straalen N.M. and Timmermans M.J.T.N. 2002; Medina *et al.* 2007; Ribeiro and Lopes 2013; Fasola *et al.* 2015), mainly through genetic drift bottlenecks, especially of small sized populations facing partially lethal pulses of contaminants, and through natural selection, both directly by eradicating the most sensitive genotypes and/or indirectly, by altering among-species interactions (e.g., patterns of predation, competition or parasitism). The irreversible loss of alleles is one of genetic erosion most serious possible consequences, unless gene flow or mutations counterbalance it (Ribeiro and Lopes 2013). A second one is the decrease of population growth rate and ultimately population extinction due to a reduction in the average fitness, through two possible pathways: an increased mutational load and tolerance associated fitness costs (Ribeiro and Lopes 2013). An increased mutation load ― drift up in frequency of slightly disadvantageous mutations ― is of particular concern in small inbred populations. Fitness costs associated to tolerance acquisition are possible, namely due to energy re-allocation from fitness enhancing functions to detoxification mechanisms (Fasola *et al.* 2015). A third possible consequence of genetic erosion is a decreased adaptive potential and, therefore, an enhanced susceptibility to future genetic erosion (Fasola *et al.* 2015); small sized populations with excessive homozigosity due to genetic drift and/or inbreeding being also of most concern (Ribeiro and Lopes 2013). Species with small sized populations (see above) and limited mobility (to ensure gene flow) are, therefore, the most threatened by genetic erosion. Amphibians fit in and, furthermore, are also facing a global decline (Barinaga 1990; Stuart *et al.* 2004; Blaustein and Bancroft 2007; O'Brien and Allentoft 2010). The present study tackled the probability of losing alleles resulting from chemical contamination in amphibian populations; testing the recessive tolerance inheritance (working-) hypothesis: "A contaminant [acting as a directional selection pressure] can eliminate alleles if [the genetic component of] tolerance is a recessive or incompletely dominant trait" is what the authors of the present work aimed at. It is worth noting that it is not a very likely hypothesis because, especially under stressful conditions, fitness tends to correlate with heterozygosity (Allendorf and Leary 1986; Hansson and Westerberg 2002; Reed and Frankham 2003; Schmeller *et al.* 2007; Luquet *et al.* 2011), i.e. one might expect that overdominance ― heterozygotes being more tolerant than either homozygote ― would be the rule. A possible explanation is that heterozygotes frequently present a higher metabolic efficiency, with consequently lower energy requirements, than homozygotes (Parsons 1997). However, this reasoning is an oversimplification, since the dominance level can change with the stressor intensity (Macnair 1997). Both tolerance as a polymorphism — clearly different phenotypes, e.g. only tolerant and sensitive — and tolerance with a polygenic basis — tolerance being a continuous variable —

have been reported (Ribeiro and Lopes 2013), and, further complicating, many minor genes may determine tolerance at mild levels of directional selection, while at very high stressor intensities only a few major genes may play a role (Hoffmann and Parsons 1991; Macnair 1997; Parsons 1997). In the present study, metal tolerance inheritance was investigated by observing the frequency of organisms with different levels of a trait subjected to a strong directional selection (i.e., a selectable marker); tolerance being the trait and acid mine drainage, at a lethal concentration, being the selective pressure. Observations were made in egg masses laid by the Perez's frog, *Pelophylax perezi*, belonging to a natural population which was previously exposed neither to acid mine drainage nor to metals. Comparing the observed patterns of frequency distribution of tolerance within egg masses with the expected distributions for each pattern of tolerance inheritance (dominant, recessive, underdominant, overdominant, and incompletely dominant) allowed to preliminarily test the recessive tolerance inheritance (working-) hypothesis.

Material and methods

Study organism:

The model organism for this study was the Perez's frog: *Pelophylax perezi* (Arnold and Ovenden 2002) (López-Seoane 1885), formerly known as *Rana perezi*. The species has a conservation status of least concern by the International Union for the Conservation of Nature (Bosch *et al.* 2013). It is endemic and common in the Iberian Peninsula and southern France, although its original northern range limit is still debated. It was introduced in Madeira, Balearic, central as well as west Canary (Arnold and Ovenden 2002) and Azores islands and in the United Kingdom (Loureiro *et al.* 2008; Bosch *et al.* 2013). It can be encountered in rivers, streams and irrigation ditches, preferring areas with low current. It is very common in ponds, marshes, and reservoirs (Arnold and Ovenden 2002; Bosch *et al.* 2013). It can tolerate temperatures up to 35°C and down to 3°C and it can be found at altitudes up to 2300 meters, in the Spanish Sierra Nevada mountains (Almeida *et al.* 2001; Bosch *et al.* 2013). The Perez's frog can colonize eutrophic and even contaminated waters (Sillero and Ribeiro 2010; Marques *et al.* 2013), making it a pioneer species in recovering habitats (Arnold and Ovenden 2002). This ability to cope with environmental stressors makes this species ideal to compare population responses between pristine and contaminated environments.

Assay setup:

During seven visits, from 13th April 2013 to 8th August 2013, 21 *P. perezi* egg masses were collected at the uncontaminated sampling site of Quinta da Boa Vista (central west coast of Portugal, 40°35'48"N – 8°41'43"W). Masses were only collected if they had been laid in the previous 24h, i.e.

containing eggs at developmental Gosner's stages 8 to 10. Eggs were collected as early as possible in Monday mornings and rapidly transported to the laboratory. Here, the masses were temporarily housed in small water aquaria (20x20x30 cm) containing Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) medium (American Society for Testing and Materials International (ASTM) 2004; Organisation for Economic Co-operation and Development (OECD) 2008). Data on the average air temperature, the amount of rain, the average wind speed, and the average insulation at each day of egg collection were obtained from the datasets of the Portuguese environmental agency Instituto Português do Mar e da Atmosfera.

Tests were performed in plastic Petri dishes (55 mm diameter) with 10 ml test solution. Each Petri dish contained 5 eggs. Eggs were exposed to a 60% dilution (with FETAX medium) of the acid mine drainage (AMD) collected at the abandoned São Domingos mine (southeast Portugal, 37°39'15"N – 7°30'31"W). Due to the ongoing oxidation of mine tailings, this AMD is very acid (pH≈2), highly rich in metals, with an electrical conductivity around 4,5 mS/cm, and isolated from other sources of contamination (Sobral *et al.* 2013). Metal concentrations were quantified in the 100% AMD and in a 10% dilution, by graphite furnace atomic absorption (Cd, Co, Ni, and Pb), by inductively coupled plasma atomic emission (Al, Cu, Fe, Mn, and Zn), and by hydride generation atomic absorption (As), in a certified laboratory (Instituto Superior Técnico, Lisbon, Portugal) (Table3.1). Because metal concentrations in the 10% dilution are approximately one tenth of those in the 100% AMD (Table 1), metal concentrations in a 60% dilution are expected to be approximately six-fold higher than those in the 10% AMD dilution; the pH in the 60% dilution remained very acid (pH≈3).

Table3.1: Metal concentrations (µg/L) in the acid mine drainage (AMD) of the São Domingos mine (southeast Portugal).

AMD	**Al**	**Fe**	**Cu**	**Mn**	**Zn**	**Co**	**Ni**	**Cd**	**Pb**	**As**
100%	$440x10^3$	$353x10^3$	$41x10^3$	$28x10^3$	$21x10^3$	$2.5x10^3$	800	308	24	1.5
10%	$46x10^3$	$33x10^3$	$4x10^3$	$2.9x10^3$	$2.1x10^3$	250	72	7.3	3	<1.0

Eggs from different egg masses were not mixed, i.e. each mass was analyzed separately. For each egg mass, there were 4 control (FETAX medium) replicates, in a total of 20 eggs; while the number of AMD exposed eggs varied depending on the mass size. The maximum number of exposed replicates per egg mass was 20 (100 eggs); 17 out of 21 masses had more than 13 replicates and the remaining 4 masses (B, E, L and O) had at least 6 replicates. The eggs' jelly coat was not removed to mimic natural scenarios. Frog embryos were placed in a conditioned chamber (23°C) under a 14h/10h light/dark photoperiod. The exposure started the day of collection (Monday) at 21h (UK time) until

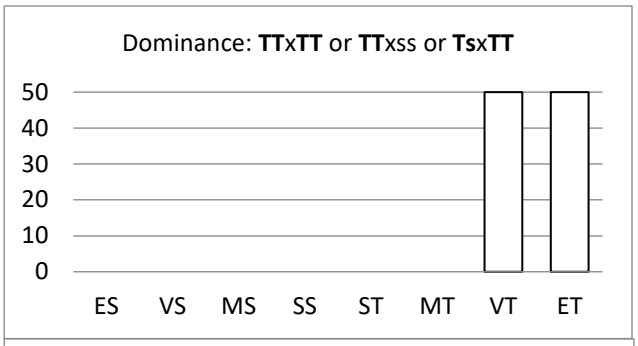
21h of the following Friday. Embryos were checked for death (at a 10x magnification using a Leica MS5 microscope) following a predetermined base 10 logarithmic time scale (by successively adding 0.15 to the $\log_{10}720$): 12h00min (=720min), 16h57min, 23h57min, 33h49min, 47h46min, 67h29min, and 95h19min. The pH (Wissenschaftlich Technische Werkstätten WTW pH330i) and conductivity (WTW conductivity440i) were measured following the same time schedule. Embryos started the assay at approximately Gosner's stages 9 to 10 and, and those who survived reached approximately Gosner's stages 21 to 22. During each assay, egg status and its development, response to gentle prodding and circulatory system functioning were sequentially inspected to assess embryo death.

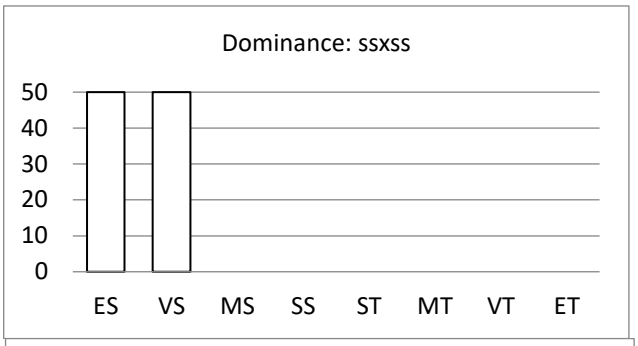
Statistical analysis:

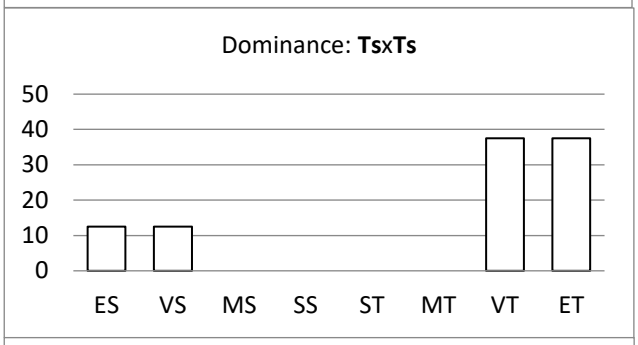
Masses were labeled with alphabet letters from A to U (in order from the most sensitive to the most tolerant, i.e. increasing tolerance). Within each egg mass, eggs were classified from extremely sensitive to extremely tolerant depending on when they were found dead, from 12h00m up to more than 95h19min. Median, lower and upper quartile of lethal time values (LT_{50}, LT_{25} and LT_{75}, respectively) of each egg mass were calculated using the PriProbit software (Sakuma 1998). An egg mass was categorized as critically sensitive when its LT_{75} was below the mean of the set of LT_{50} values and as safely tolerant when its LT_{25} was above the mean LT_{50} (Ribeiro and Lopes 2013). The relative spread value for each egg mass was computed as the interquartile distance – LT_{75} minus LT_{25} – divided by its median – the LT_{50} (Ribeiro and Lopes 2013). The following analyses were performed using Statistica for Windows 7.0 (StatSoft, Aurora, CO, USA). Values of LT50 versus their respective relative spreads were checked for parametric correlation. To check for the influence of environmental parameters on tolerance, correlations of rank of the collection day (1 to 7), average air temperature, amount of rain, average wind speed, average insulation at each day of egg collection, versus the respective LT_{50} values were performed.

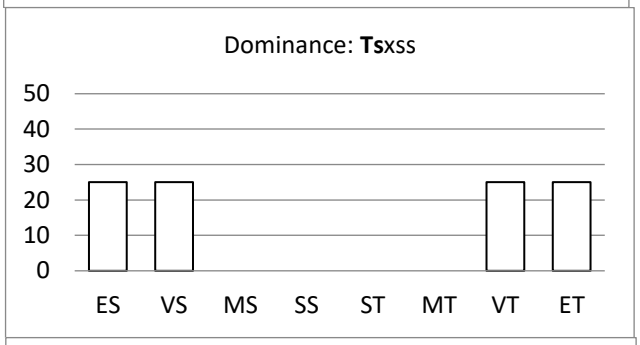
Genetic mechanism evaluation:

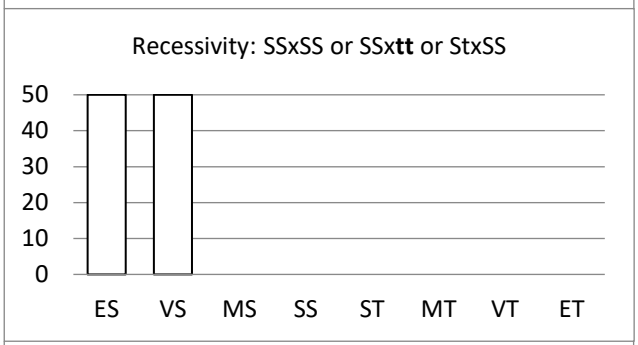
To investigate the inheritance mechanism of the tolerance to lethal levels of AMD (as a dominant, recessive, overdominant, underdominant, or incompletely dominant trait), graphs were prepared showing the expected patterns of F1 tolerance results for the simplest possible scenario: tolerance being a trait determined by a single gene with only two alleles (Fig.3.1). The degree of dominance of the tolerance trait is 50%, meaning that a heterozygote presents a median tolerance. The eggs' sensitivity distribution of an egg mass would shift to the right or to the left if the degree of dominance differs from 50%, from almost full recessivity (dominance degree slightly above 0%) to almost full dominance (dominance degree slightly below 100%).

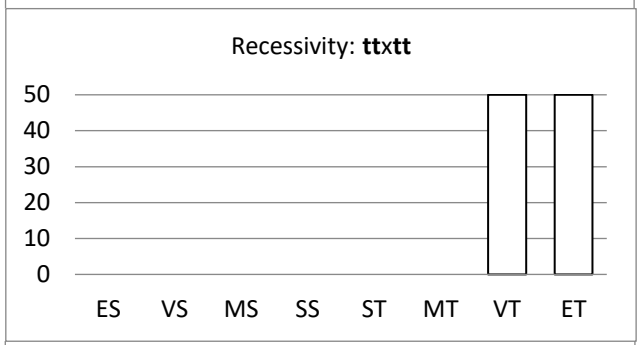

Dominance: **TT**x**TT** or **TT**xss or **Ts**x**TT**
50
40
30
20
10
0
ES VS MS SS ST MT VT ET

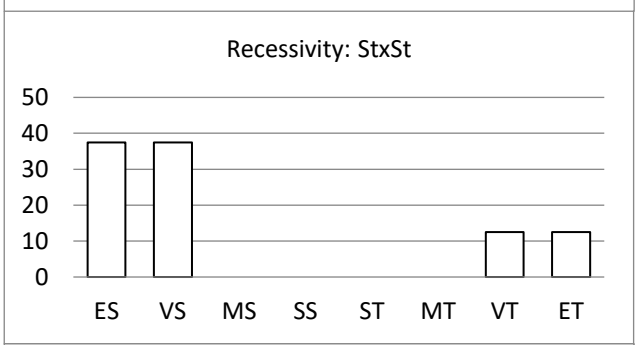

Dominance: ssxss
50
40
30
20
10
0
ES VS MS SS ST MT VT ET

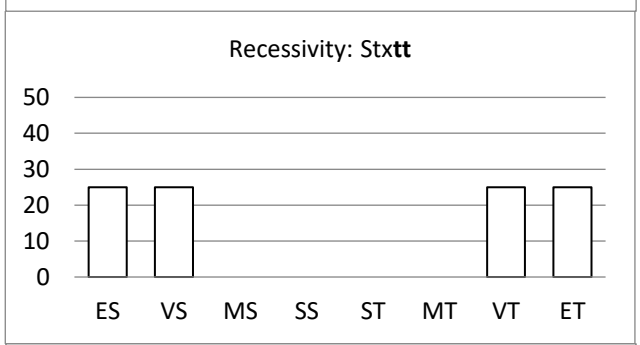

Dominance: **Ts**x**Ts**
50
40
30
20
10
0
ES VS MS SS ST MT VT ET

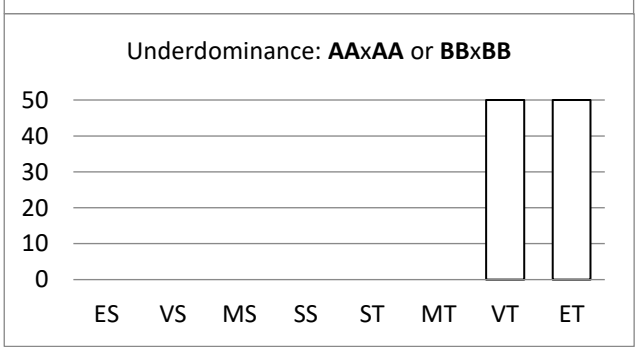

Dominance: **Ts**xss
50
40
30
20
10
0
ES VS MS SS ST MT VT ET

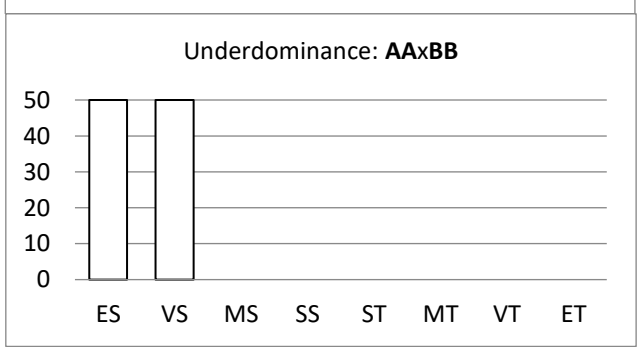

Recessivity: SSxSS or SSx**tt** or StxSS
50
40
30
20
10
0
ES VS MS SS ST MT VT ET


Recessivity: **tt**x**tt**
50
40
30
20
10
0
ES VS MS SS ST MT VT ET


Recessivity: StxSt
50
40
30
20
10
0
ES VS MS SS ST MT VT ET


Recessivity: Stx**tt**
50
40
30
20
10
0
ES VS MS SS ST MT VT ET


Underdominance: **AA**x**AA** or **BB**x**BB**
50
40
30
20
10
0
ES VS MS SS ST MT VT ET


Underdominance: **AA**x**BB**
50
40
30
20
10
0
ES VS MS SS ST MT VT ET

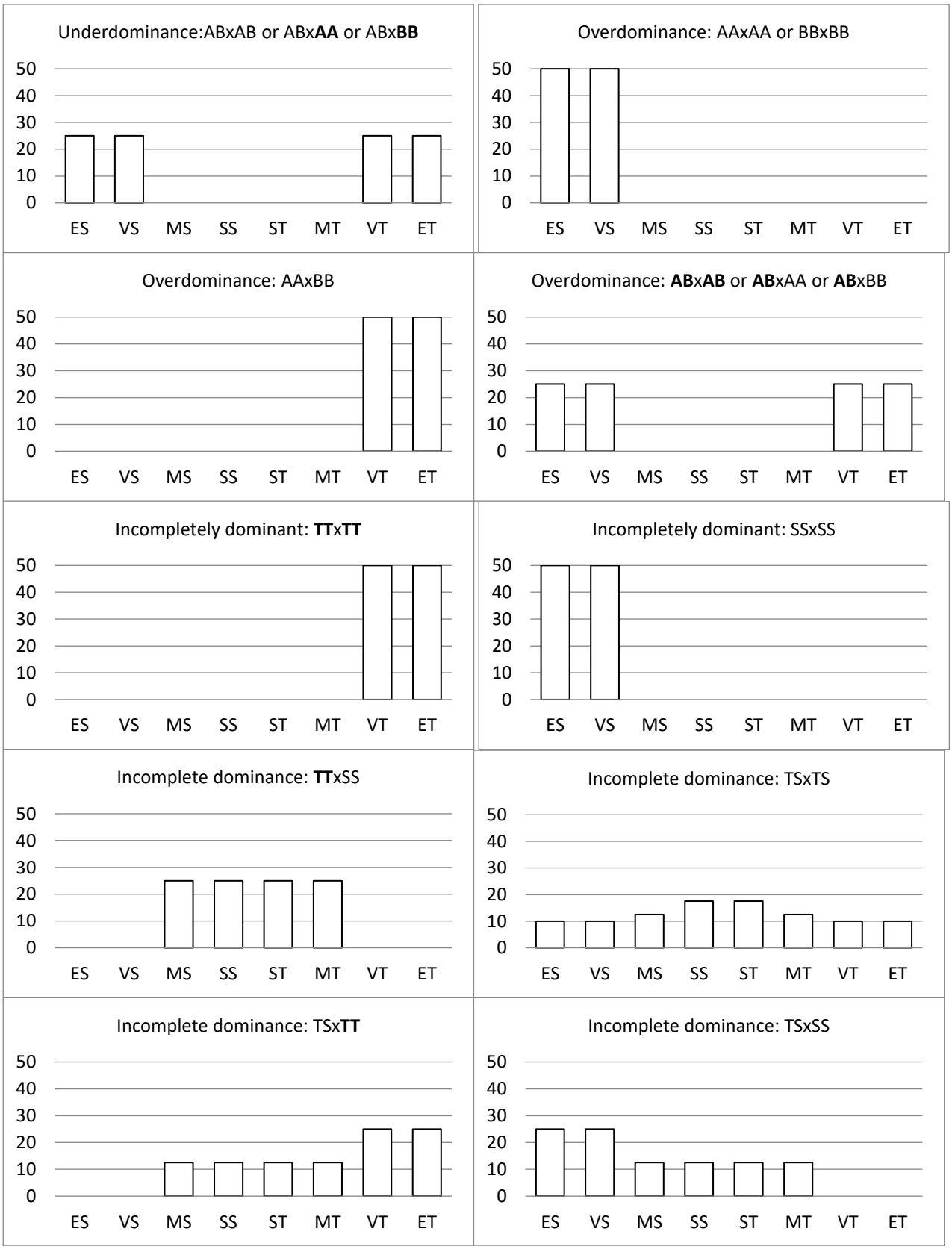


Fig.3.1: Expected theoretical frequencies (in %) of eggs (F1 generation), of Perez's frog (Pelophylax perezi) egg masses, belonging to eight classes of lethal tolerance to a 60% dilution of acid mine drainage, in all possible scenarios of inheritance mechanism (dominance, recessivity, underdominance, overdominance, and incomplete dominance) from all possible parental genotype crossings in a two-alleles system (T and S) of a single gene. The classes correspond to the eight observation moments (following a logarithmic time scale from 12h00min up to 95h19min of exposure). T - allele conferring tolerance. S - allele not conferring tolerance. In the case of overdominance and underdominance, where there

is not really an allele conffering tolerance, they were indicate as alleles A and B. Lower case indicates the recessive allele. Bold face indicates the most tolerant genotypes. The X-axis represents time in subsequent observations. ES, VS, MS, SS, ST, MT, VT, and ET – Extremely, Very, Moderately, and Slightly Sensitive or Tolerant eggs, respectively.

Results

All assays were considered valid since mortality in the controls never exceeded 20%. This value was reached in four out of 21 egg masses. Environmental parameters neither at each collection day (rank along the breeding season, average air temperature, amount of rain, average wind speed, and average insulation at each day of egg collection) nor during testing (pH and conductivity) were correlated with LT_{50} values ($p>0.05$). The sampled population presented a broad range of egg mass tolerance to AMD, from critically sensitive (masses from B to J) to safely tolerant (T and U) egg masses, with LT_{50} values covering over one order of magnitude (Table 3.2, Fig.3.2 and 3.3).

Table.3.2: Median lethal time values (LT_{50}) with their 95% confidence limits and respective relative spread of the 21 Perez's frog egg masses exposed to a sample of acid mine drainage. The average of all LT_{50} values is also indicated.

Egg Mass	LT_{50} (minutes)	LT_{50} 95% conf. limits	REL. SPREAD
A	312.12	61.85 – 878.36	627.24
B	369.30	46.00 – 646.07	150.29
C	483.21	323.65 – 583.23	66.57
D	518.82	383.04 – 611.52	70.06
E	524,31	383.39 – 651.17	158.64
F	533.51	395.04 – 689.52	61.16
G	690.55	579,04 – 792.65	166.21
H	760.90	760.90 – 760.90	39.00
I	809.99	572.32 – 1226.08	82.55
K	883.14	1309,58 – 2002.27	89.00
J	1034.86	936.07 – 1130.22	95.33
L	1309.58	1200.23 – 1417.85	97.96
M	1475.26	1241.11 – 1712.82	97.40
N	1615.01	1457.44 – 1751.36	129.83
O	1648.43	1166.15 – 2093.03	170.96
P	1670.49	1309.58 – 2002.27	146.54
Q	1972.70	1533.19 – 2336.57	152.05
R	2129.63	1645.71 – 2566 52	259.86
S	2151.56	1645.71 – 2566.52	147,99
T	7155.24	3281.15 – 9112.73	162.87
U	13239.40	2962.91 – 22109.80	375.08

Mean LT_{50} (minutes)
1966.10

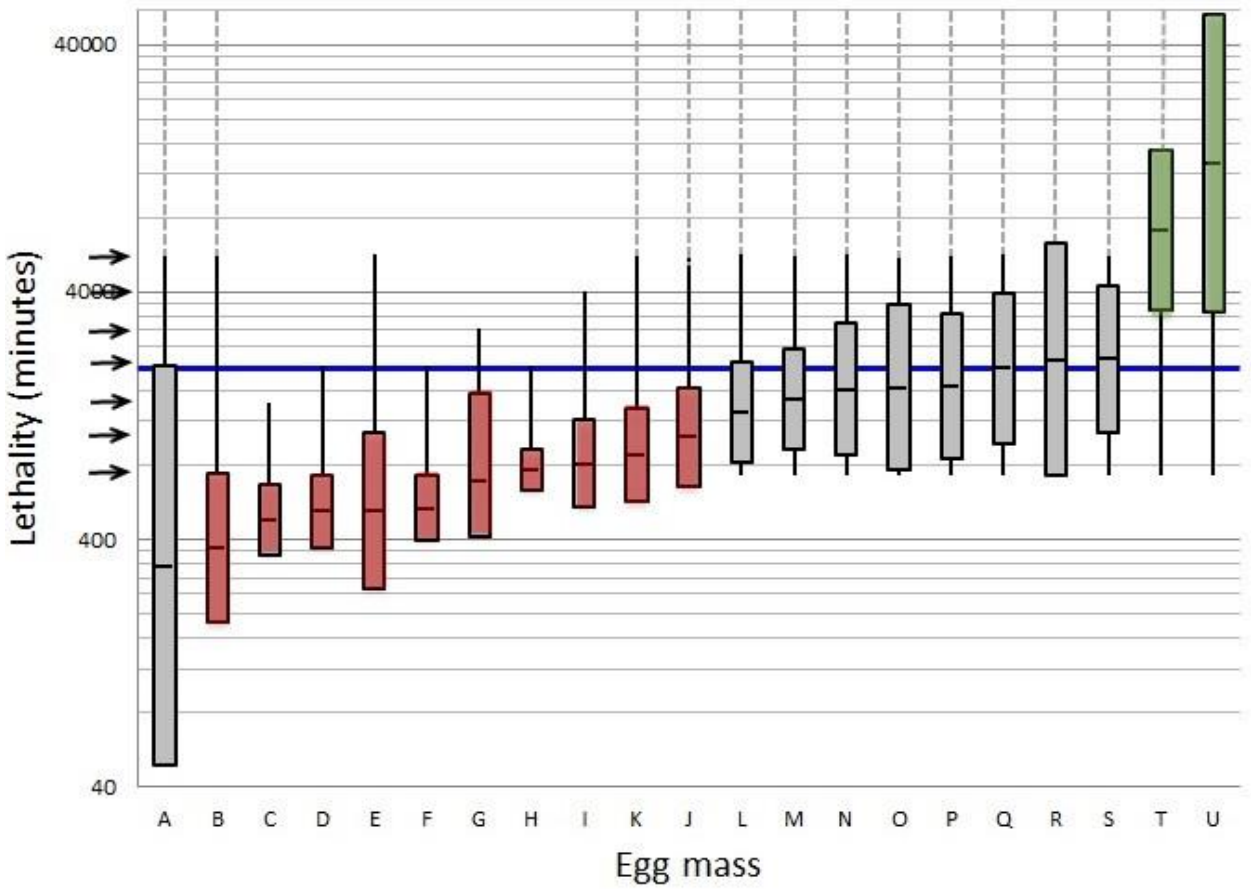
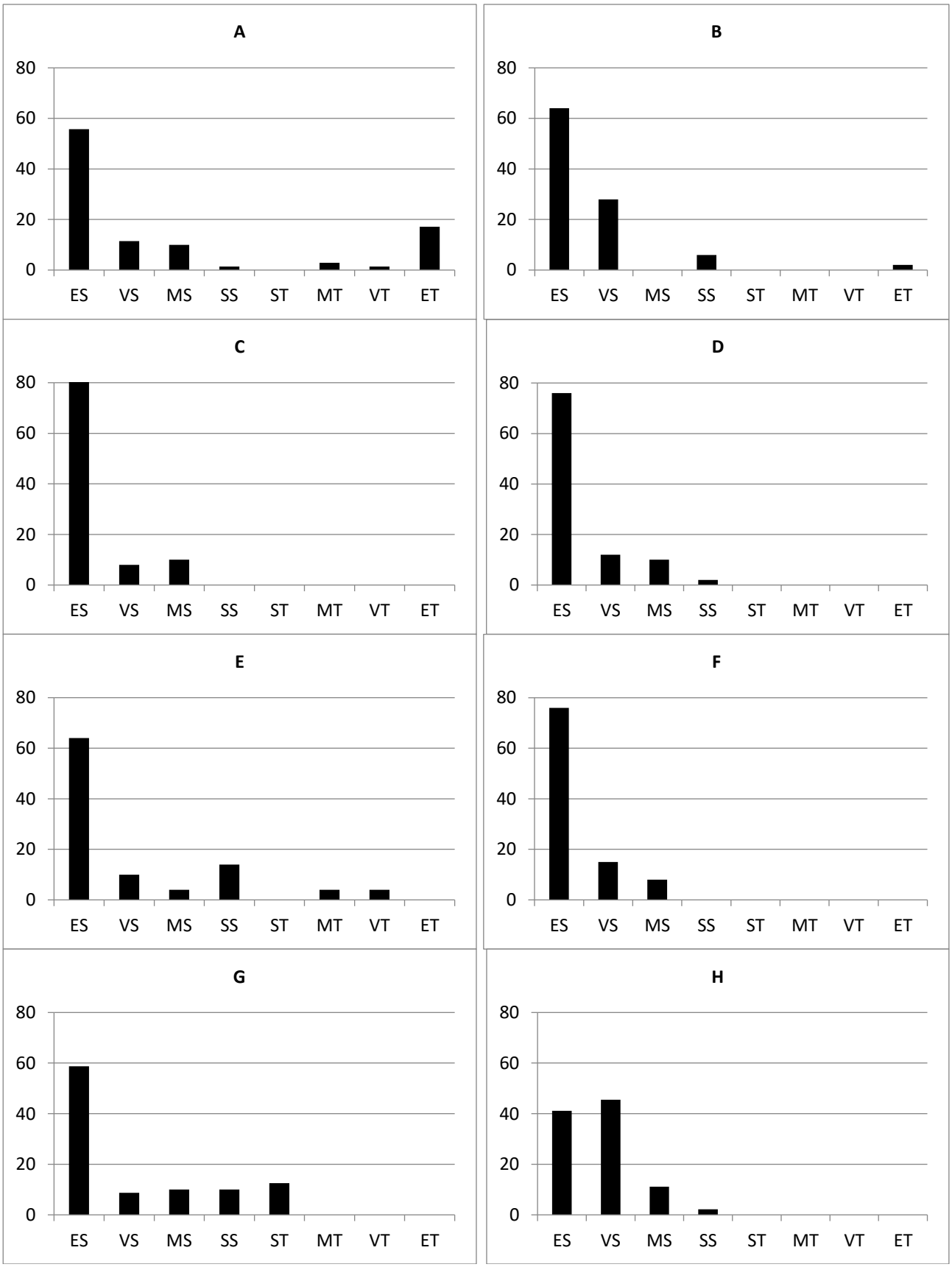
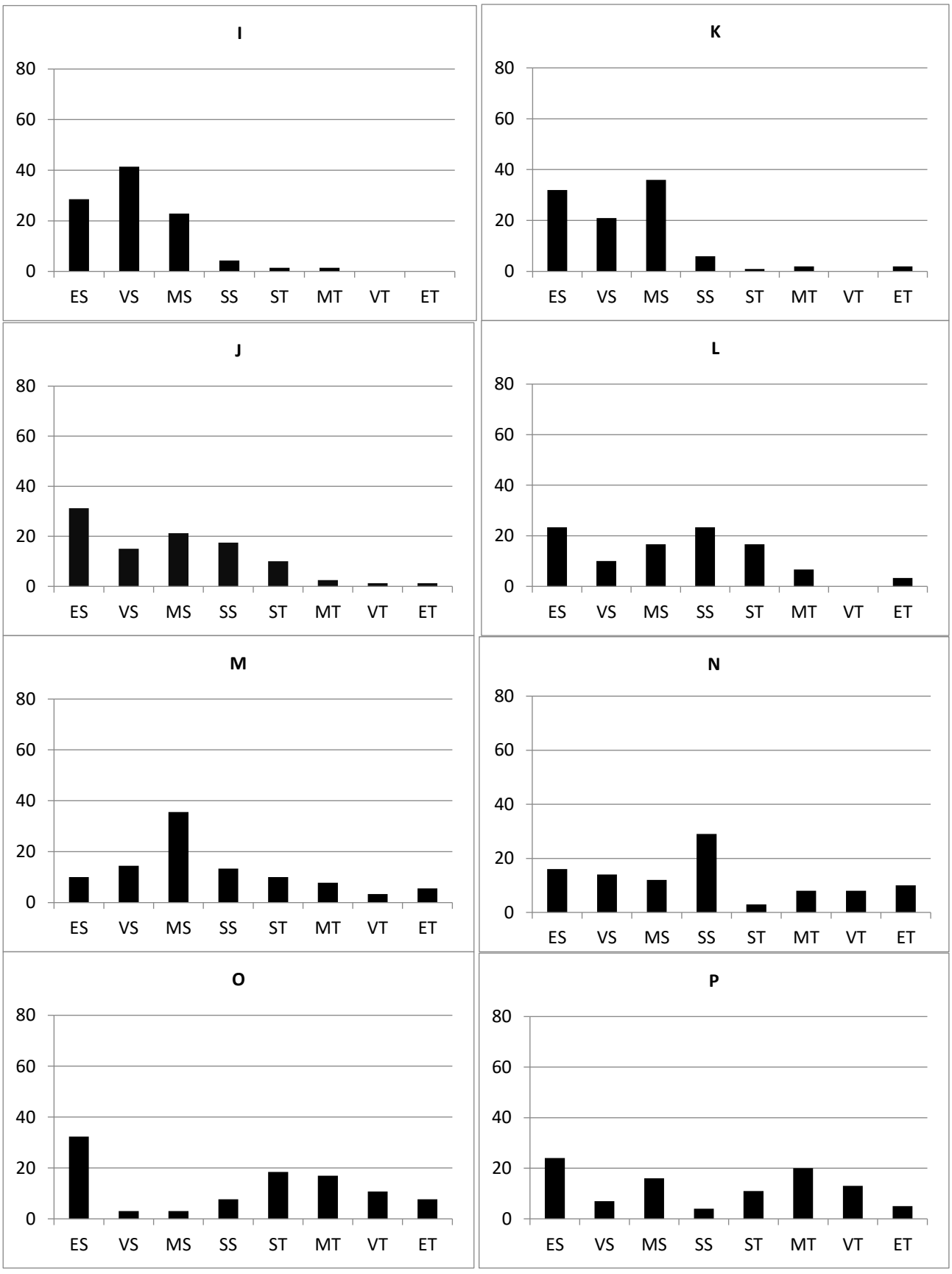


Fig.3.2: Box plots representing the median, the lower and the upper quartiles of lethal time values (exposure times after which 50, 25 and 75% of eggs died) of eggs within each of 21 Perez's frog (*Pelophylax perezi*) egg masses (A to U), collected in a reference pond, exposed to a 60% dilution of metal-rich acid mine drainage. Vertical black lines represent maximum and minimum lethal time values for each egg mass (until the last observation made). Grey dashed lines indicate some eggs were still alive at the end of the last observation. The thick horizontal blue line represents the average of the 21 median lethal time values. Observations, indicated by arrows, were made 720, 1017, 1436, 2029, 2866, 4048, and 5719 minutes after the start of the assay.


A
B
C
D
E
F
G
H
80
60
40
20
0
ES
VS
MS
SS
ST
MT
VT
ET


I
80
60
40
20
0
ES VS MS SS ST MT VT ET
K
80
60
40
20
0
ES VS MS SS ST MT VT ET
J
80
60
40
20
0
ES VS MS SS ST MT VT ET
L
80
60
40
20
0
ES VS MS SS ST MT VT ET
M
80
60
40
20
0
ES VS MS SS ST MT VT ET
N
80
60
40
20
0
ES VS MS SS ST MT VT ET
O
80
60
40
20
0
ES VS MS SS ST MT VT ET
P
80
60
40
20
0
ES VS MS SS ST MT VT ET

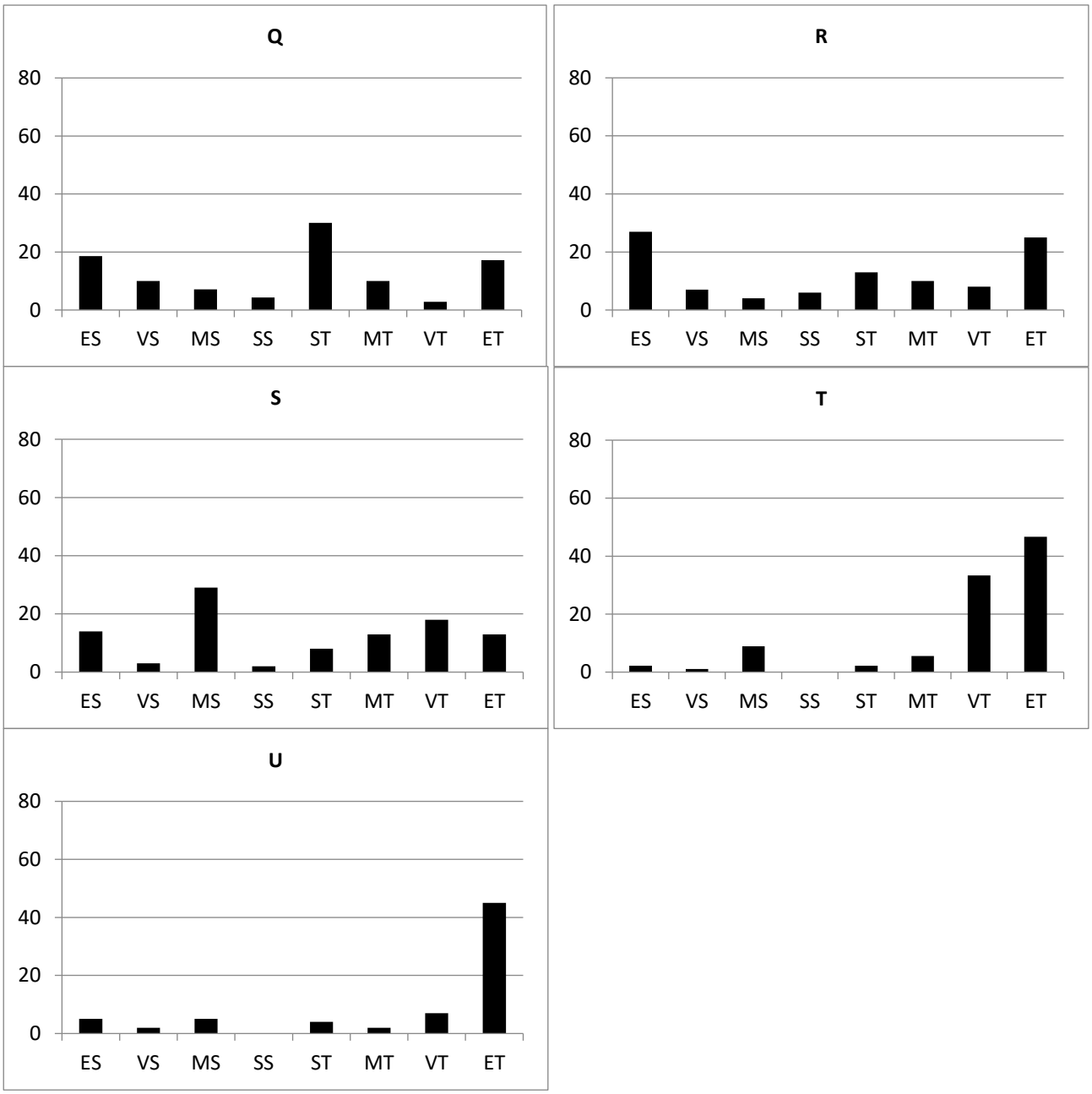


Fig.3.3: Frequencies (in %) of eggs belonging to eight classes of lethal tolerance to a 60% dilution of acid mine drainage, in each of 21 Perez's frog (*Pelophylax perezi*) egg masses (A to U), collected in a reference pond. The classes correspond to the eight observation moments (following a logarithmic time scale from 12h00min up to 95h19min of exposure). The X-axis represents time in subsequent observations. ES, VS, MS, SS, ST, MT, VT, and ET – Extremely, Very, Moderately, and Slightly Sensitive or Tolerant eggs, respectively.

All egg masses had relative spread values below 300% with two exceptions: masses A and U (the most sensitive and the most tolerant respectively) (Table 3.2 and Fig.3.4). Mass A, with a relative spread over 600% was the only egg mass presenting extremely sensitive and extremely tolerant eggs as the most frequent classes (Fig.3.3). Furthermore, mass A also showed almost no intermediately tolerant eggs. A significant association between egg mass tolerance (LT_{50}) and the within egg mass variability (relative spread) was not found ($p>0.01$) and no clear pattern was observed (Fig.3.4).

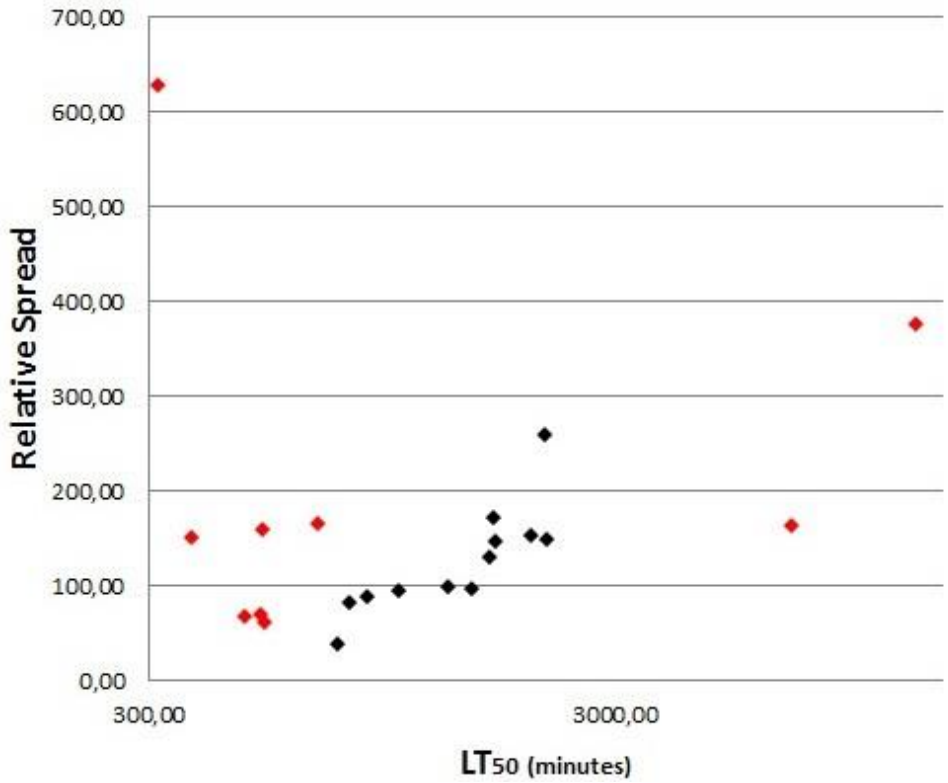


Fig.3.4: Relative spread (difference between the upper and lower quartiles relatively to the median) of egg tolerance to a 60% dilution of metal-rich acid mine drainage of 21 Perez's frog (*Pelophylax perezi*) egg masses, collected in a reference pond, versus the respective median lethal time values (LT50 – exposure time after which 50% of eggs died). Red points indicate when the calculated LT50 had a value lower than the time in which we performed the first observation (720 minutes) or higher than the last observation (5719 minutes).

Discussion and conclusions

The low mortality of the controls eggs, in the present work suggests that intrinsic mortality rate (under optimal environmental conditions) of *P. perezi* egg masses, in the field, is very low or even zero. Therefore, mortality in the field could be assumed to be the result of environmental perturbations (for example contamination, predation or other environmental pressures). In controlled laboratory conditions there was high variability, between egg masses, in the tolerance to AMD. These responses, following exposure to AMD, did not variate in function of pH or conductivity. The observed variability was neither function of the environmental conditions at the moment of the egg mass spawning. Median lethal time values showed neither a trend during the breeding season nor any association with environmental parameters. Therefore, it can reasonably be assumed that differences in tolerance among egg masses were almost exclusively due to genetic determination. Another reasonable assumption is that each egg mass was fertilized by a one male only. However, field herpetologists have reported *amplexi* involving more than one male, which theoretically might result in egg masses with more than one father and, thus, with a possibly broader genetic makeup (Lodé and Lesbarrères 2004). Such a situation in the present study could lead to an egg mass with a relative

spread much larger than all other egg masses, which matches egg mass A (Fig.3.3). Such a different outcome could be partially explained by some pre-copulatory competition between males, in which the first or the last male involved in the mating gets advantage in fathering the majority of the eggs. The very large relative spread, for mass A, resulted from the presence of extremely tolerant and extremely sensitive eggs, with almost no intermediately tolerant eggs (Fig.3.3). This U-shaped bimodal distribution pattern of egg tolerance could also be determined by tolerance being a trait other than incompletely dominant (Fig.3.1). However, besides egg mass A, no other egg mass presented this pattern. Indeed, although 14 out of the 20 other egg masses also included eggs at both extremes of tolerance, bimodality is clearly the exception rather than the rule (Fig.3.3), supporting incomplete dominance as the most probable inheritance mechanism of tolerance and ruling out dominance, recessivity, underdominance, or overdominance (Fig.3.1). Even if egg tolerance to lethal levels of AMD has a polygenic basis and even if implicated genes have more than two alleles, this deduction in all likelihood remains valid for the trait as a whole, although the probability of losing alleles may be much lower.

To fully support the worst-case scenario of the recessive tolerance inheritance (working-) hypothesis – (full) recessivity – each egg mass should fall in one the three following categories: (i) all eggs being sensitive (with a small relative spread; approximately matched by egg masses C, D, F, and H), resulting from one parent being homozygous dominant, (ii) all eggs being tolerant (also with a small relative spread; matched by no egg mass), resulting from homozygous recessive parents, or (iii) eggs being either tolerant or sensitive (with a large relative spread; matched by egg mass A), resulting from the crossing of an heterozygous with either a homozygous recessive or another heterozygous individual (Fig.3.1). A partially lethal input of AMD would eliminate the most sensitive genotypes, consequently wiping out the dominant allele. In the present study, only six out of 21 egg masses were found to match tolerance to AMD as a (fully) recessive trait (Fig.3.3). Furthermore, under this scenario of (full) recessivity, relative spreads of intermediately tolerant egg masses should be clearly higher than those of both sensitive and tolerant egg masses, resulting in an inverted U-shaped association of relative spread versus tolerance, which was not found (Fig.3.4).

The other considered scenario in the original formulation of the hypothesis (Ribeiro and Lopes 2013) – incomplete dominance – was almost fully supported by our observations: egg masses presented, in general, broad ranges of egg tolerance with a unimodal distribution (with intermediate relative spread values) (Fig.3.3). The egg mass A was a clear exception (Fig.3.1 and 3.3).

If tolerance is confirmed as an incomplete dominant trait then a partially lethal input of AMD would eliminate the most sensitive genotypes, but not fix in the population the allele conferring tolerance. That would happen solely under an exposure to an almost fully lethal concentration of AMD, leaving

only the most tolerant genotypes, which would be the homozygous tolerant ones. If tolerance is inherited as a (fully) dominant, overdominant or underdominant trait, then neither a partially lethal nor even an almost fully lethal input of AMD would eliminate alleles. This is so because either the heterozygote would be maximally tolerant (dominance and overdominance) or both homozygotes were the most tolerant individuals (underdominance). As with (full) recessivity, only six (egg masses A, C, D, F, H, and I) out of 21 egg masses could support (full) dominance, overdominance or underdominance (Fig.3.1 and 3.3).

In the present study, the above conclusions were drawn according to a genetic system in which a single gene with two alleles was imagined. Such an assumption is plausible because previous studies reported only a few major genes determining the tolerance to very intense selective pressure (Hoffmann and Parsons 1991; Macnair 1997; Parsons 1997). For more complex systems of genetic determination, such as more than two alleles at a single gene conferring a gradient of tolerance, broad ranges of egg tolerance with a unimodal distribution (with intermediate relative spread values), as observed in the present study, would also result from incomplete dominance. Even for a scenario of a polygenic basis, the results in the present study point to the possibility of an incomplete dominance occurrence, at least at some genes, which would result in a risk of alleles' loss.

The observed low occurrence of safely tolerant egg masses (T and U) compared with those critically sensitive (masses from B to J), in the sampled frog population, could be explained by the lack of past selection for tolerance to acid mine drainage. Indeed, genetically determined tolerance to chemical stress may result in fitness costs, emerging from physiological processes, through energy re-allocation from fitness enhancing functions to detoxification mechanisms, and from genetic processes, through antagonistic pleiotropy and epistasis (Hoffmann and Parsons 1991; Shirley and Sibley 1999; Klerks *et al.* 2011). Therefore, tolerant individuals would only be in advantage when the population is exposed to contamination.

In conclusion, the results of the present study seem to preliminarily support the recessive (or incompletely dominant) tolerance inheritance (working-) hypothesis (Ribeiro and Lopes 2013), highlighting the need to further address and prevent contaminant-driven genetic erosion, also because of the possible irreversible loss of alleles, especially in small populations as those of amphibians.

Aknowledgments

This work was supported by funding FEDER through COMPETE - Programa Operacional Factores de Competitividade and by FCT Fundação para a Ciência e Tecnologia through project: (PTDC/BIA-BIC/3488/2012), within the CESAM's strategic programme (UID/AMB/50017/2013) and the doctoral fellowship of Emanuele Fasola (SFRH/BD/88955/2012). The authors would like to thank: Bárbara Santos, Nuno Costa, Sara Costa, Ines Bulhosa and Nuno Martins for their support during fieldwork; Bruno Costa for his support with statistical analysis, as well as Abel Ferreira for being helpful during lab work.

References

ALLENDORF, F. W.; LEARY, R. - Heterozygosity and fitness in natural populations of animals. In **Conservation biology: the science of scarcity and diversity.** [S.l.], Soule M.E. (ed). Conservation Biology. Sinauer: Sunderland, MA, 1986. p. 75–76.

ALMEIDA, N.; ALMEIDA, P.F.; GONÇALVES, H.; SEQUEIRA, F.; TEIXEIRA, J.; ALMAIDA, F.F.- **Guia FAPAS dos anfíbios e répteis de Portugal.** Porto, Portugal : FAPAS, 2001

AMERICAN SOCIETY FOR TESTING AND MATERIALS INTERNATIONAL (ASTM) - Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - Xenopus (FETAX). (2004) 1439–98.

ARNOLD, Nicolas; OVENDEN, Denys - **Reptiles and Amphibians of Britain and Europe.** [S.l.] Collins; New edition, 2002

BARINAGA, M. - Where have all the froggies gone? **Science**. 2 (1990) 1033–1034.

BLAUSTEIN, AR; BANCROFT, BA - Amphibian population declines: evolutionary considerations. **BioScience**. 57:5 (2007).

BOSCH, J. - *Pelophylax perezi***.** (2013). Available at WWW:<URL:www.iucnredlist.org>.

CBD - Decision adopted by the conference of the parties to the Convention on Biological Diversity at its tenth meeting. CONFERENCE OF THE PARTIES TO THE CONVENTION ON BIOLOGICAL DIVERSITY. Tenth meeting Nagoya, Japan (2010).

FASOLA, E.; RIBEIRO, R.; LOPES, I. - Microevolution due to pollution in amphibians: A review on the genetic erosion hypothesis. **Environmental Pollution**. ISSN 02697491. 204:2015) 181–190. doi: 10.1016/j.envpol.2015.04.027.

HANSSON, B.; WESTERBERG, L. - On the correlation between heterozygosity and fitness in natural populations. **Molecular Ecology**. (2002).

HOFFMANN, A. A.; PARSONS, P. A. - **Evolutionary genetics and environmental stress.** Oxford, UK ed. [S.l.] : Oxford University Press (1991).

KLERKS, PL; XIE, Lingtian; LEVINTON, JS - Quantitative genetics approaches to study evolutionary processes in ecotoxicology; a perspective from research on the evolution of resistance. **Ecotoxicology**. 2011) 513–523. doi: 10.1007/s10646-011-0640-2.

LODÉ, T.; LESBARRÈRES, D. - Multiple paternity in Rana dalmatina, a monogamous territorial breeding anuran. **Naturwissenschaften**. 91:1 (2004) 44–47. doi: 10.1007/s00114-003-0491-7.

LOUREIRO, A.; ALMEIDA, N.F.; CARRETERO, M.A.; PAULO, O.S.- **Atlas de Anfíbios e Répteis de Portugal.** [S.l.] : INOVA, Portugal., 2008

LUQUET, E.; DAVID, P; LENA, J; JOLY, P; KONECNY, L; DUFRESNES, C; PERRIN, N; PLENET, S - Heterozygosity-fitness correlations among wild populations of European tree frog (*Hyla arborea*) detect fixation load. **Molecular ecology**. ISSN 1365-294X. 20:9 (2011) 1877–1887. doi: 10.1111/j.1365-294X.2011.05061.x.

MACNAIR, M. N. - The evolution of plants in metal-contaminated environments. In VERLAG, BIRKHAUSER (Ed.) - **Environmental Stress. Adaptation and Evolution, by Bijlsma and Loeschcke.** Basel, Switzerland : [s.n.]. p. 3–24.

MARQUES, Sérgio M.; CHAVES, Sandra; GONÇALVES, Fernando; PEREIRA, Ruth - Differential gene expression in Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine. **Ecotoxicology and environmental safety**. . ISSN 1090-2414. 87 (2013) 115–9. doi: 10.1016/j.ecoenv.2012.10.010.

MEDINA, MH; CORREA, JA; BARATA, Carlos - Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. **Chemosphere**. 67 (2007) 2105–2114. doi: 10.1016/j.chemosphere.2006.12.024.

O'BRIEN, John; ALLENTOFT, Morten E. - Global Amphibian Declines, Loss of Genetic Diversity and Fitness: A Review. **Diversity**. ISSN 1424-2818. 2:1 (2010) 47–71. doi: 10.3390/d2010047.

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT (OECD) - **OECD Guidline for the testing of chemicals**. (2008) 1–33.

PARSONS, P. A. - Stress-resistance genotypes, metabolic efficiency and interpreting evolutionary change. In VERLAG, BIRKHAUSER (Ed.) - **Environmental Stress. Adaptation and Evolution, by Bijlsma R. and Loeschcke V.** Basel, Switzerland : [s.n.]. (1997) p. 291–305.

REED, D. H.; FRANKHAM, R. - Correlation between fitness and genetic diversity. **Conservation Biology**. 17:1 (2003) 230–237.

RIBEIRO, Rui; LOPES, Isabel - Contaminant driven genetic erosion and associated hypotheses on alleles loss, reduced population growth rate and increased susceptibility to future stressors: an essay. **Ecotoxicology (London, England)**. ISSN 1573-3017. 22:5 (2013) 889–99. doi: 10.1007/s10646-013-1070-0.

SAKUMA, M. - Probit analysis of preference data. **Applied Entomology and Zoology**. 33:339–347 (1998).

SCHMELLER, DS; SCHREGEL, Julia; VEITH, Michael - The importance of heterozygosity in a frog's life. **Naturwissenschaften**. 2007) 360–366. doi: 10.1007/s00114-006-0205-z.

SHIRLEY, M. D. F.; SIBLEY, R. - Genetic basis of a between environment trade-off involving resistance to cadmium in *Drosophila melanogaster*. **Evolution**. 53 (1999) 836–838.

SILLERO, N.; RIBEIRO, R. - Reproduction of *Pelophylax perezi* in brackish water in Porto (Portugal). **Herpetology Notes**. 3:2010) 337–340.

SOBRAL, Olímpia; MARIN-MORALES, Maria Aparecida; RIBEIRO, Rui - Could contaminant induced mutations lead to a genetic diversity overestimation? **Ecotoxicology**. ISSN 09639292. 22:5 (2013) 838–846. doi: 10.1007/s10646-013-1079-4.

STUART, SN; CHANSON, JS; COX, NA - Status and trends of amphibian declines and extinctions worldwide. **Science**. 306 (2004) 1783–1786.

VAN STRAALEN N.M.; TIMMERMANS M.J.T.N. - Genetic variation in toxicants-stressed populations: An evaluation of the «Genatic Erosion» Hypothesis. **Human and Ecological Risk Assessment: An International Journal.** 8:2 (2002) 983-1002.

CHAPTER 4.

Testing the recessive tolerance inheritance (working-) hypothesis: Frog egg masses exposed to copper.

Testing the recessive tolerance inheritance (working-) hypothesis: Frog egg masses exposed to copper.

Abstract

Contamination-induced genetic erosion in amphibian populations could determine the irreversible loss of alleles, decrease population growth rate by fitness costs and increase the susceptibility to future genetic erosion by reducing adaptability to other stressors. When the chemical tolerance is a fully (or incompletely dominant) trait – the recessive tolerance inheritance (working-) hypothesis; *sensu* Ribeiro and Lopes (Ribeiro and Lopes, 2013) – the loss of the most sensitive genotypes – contaminant-driven genetic erosion – could easily and irreversibly eliminate alleles from the impacted population. This work investigated the inheritance of tolerance to copper sulphate, in *Pelophylax perezi* (the Perez's frog). Forty egg masses, in Gosner stage 8-10, were collected from a reference site, during two different sampling seasons; 20 in 2014 and 20 in 2016. Those egg masses were exposed to a single concentration of copper sulphate (9 mg/L) plus to a control (FETAX). During the toxicity assays, time to death was recorded for each egg. At the end of the assays, the median lethal time (LT_{50}) and respective quartiles (LT_{25} and LT_{75}) were computed for each egg mass. Critically sensitive egg masses (categorized as those with an LT_{75} similar or below the average of the LT_{50} for all egg masses) were identified. The within egg mass variability in time to death responses was evaluated through its relative spread. The results suggest that genetically determined tolerance could be driven by incomplete dominance.

Keywords: Tolerance inheritance, genetic erosion, *Pelophylax perezi*, copper, incomplete dominance.

Introduction

Understanding the tolerance of natural populations to stressors is of particular concern for conservation purposes. Acting as a directional selective pressure, a stress increase can result in the disappearance of sensitive genotypes, mainly if the phenotypic plasticity of those genotypes is low (Fasola *et al*., 2015; Ribeiro and Lopes 2013; Venâncio *et al*., 2016). Researchers have discussed if genetically determined tolerance results from few major genes or from many little genetic contributions. Both cases have been reported and were also related to the intensity of the selective pressure (Barata *et al.*, 1998; Hoffmann and Parsons, 1991; Macnair, 1991). Under mild pollutant's concentrations, causing directional selection, tolerance tends to have a polygenic basis, with many

minor genes involved, while under intense pollution pulses (hard directional selection) only a few genes tend to play a central role (Hoffmann and Parsons, 1991; Macnair, 1991, 1997). Standard population genetic theory suggests that adaptation should normally be achieved by the spread of many genes each of small effect (polygenes) and that adaptation by major genes should be unusual (Hoffmann and Parsons, 1991; Macnair, 1991, 1997). However, the adaptation to contaminants and the response to anthropogenic impacts has frequently resulted from the spread of major genes (Jagadeesan *et al.*, 2014; Haridi, 1974; Hoffmann and Parsons, 1991; Hoffman and Willi, 2008; Macnair, 1991; Tabashnik, 1991). An example is that of the tolerance to DDT, in the common house fly, *Musca domestica*, which is probably governed by a single incompletely dominant gene (Lovell and Kearns, 1959; Tabashnik, 1991), as well as in the *Anopheles gambiae* complex (Haridi, 1974). This has been shown for many other insecticides as well (Wood, 1981). Emblematic is the case of the rusty grain beetle, in which the tolerance to the organophosphorous pesticide posphine is inherited and expressed as an incompletely recessive trait (Jagadeesan *et al.*, 2014). However, there is some dominant factors that comes into play only at higher pesticide concentrations (Jagadeesan *et al.*, 2014). Famous examples, in vertebrates, are related to rodenticide studies, once again showing that tolerance was determined by a single gene (Greaves *et al.*, 1976; Wallace and Mac Swiney, 1976). The same was true for tolerance to dioxin-like compounds in fish (Proestou *et al.*, 2014; Wirgin *et al.*, 2011). Genetic variability in tolerance to trace metal was also showed to be inherited by single major genes (Barata *et al.*, 1998; Macnair, 1989, 1991, Hoffmann and Parsons, 1991; Schat and Ten Bookum, 1992;), as has been studied, for example, in marine invertebrates (Gahan *et al.*, 2001). The present study focused on metal contamination, which has big impacts on the environment and can be due to many sources, like industrial processes, water acidification or mining activities (Linder and Grillitsch, 2000; Knasmüller *et al.*, 1998; Nadal *et al.*, 2004; Roark and Brown, 1996; Santoro *et al.*, 2008; Young and Harvey, 1991). Among other metal ions, copper is a concerning pollutant; it is released in the atmosphere by the combustion of fossil fuels; copper ions reach soils and water due to mining, metal alloys production and enter freshwaters after agriculture fertilizer application (Adlassnig *et al.*, 2013; Di Toro *et al.*, 2001; Lu *et al.*, 2006). In this context, discovering the genetics of tolerance to metal, especially copper, is very important. Metal pollution itself can alter the genetic variability of populations (Fasola *et al.*, 2015; Ribeiro and Lopes, 2013), thorough genetic erosion. For example, the study by Roark and Brown, showed higher proportions of heterozygous genotypes in *Gambusia affinis*, *Pimephales notatus* and *Fundulus notatus* populations of a river receiving acid mine drainage, compared to reference populations, suggesting directional selection (Roark and Brown, 1996). Furthermore Mussali-Galante et al. discovered a significant negative relationship between genetic diversity and metal concentration in the little rodent *Peromyscus melanophrys*

(Mussali-Galante *et al.*, 2013). Indeed, contamination-induced genetic erosion could be a major treat (Fasola *et al.*, 2015; Medina *et al.*, 2007; Ribeiro and Lopes, 2013; Van Straalen and Timmermans, 2002; Venâncio *et al.*, 2016). The International Convention on Biodiversity highlighted the importance to: "Improve the status of biodiversity by safeguarding ecosystems, species and genetic diversity"; specifying the importance to develop strategies for minimizing genetic erosion and safeguarding populations' genetic diversity (CBD, 2013). Genetic erosion can result from directional selection eliminating sensitive genotypes or from bottlenecking small populations with potential loss of genotypes and alleles by genetic drift (Allendorf and Leary, 1986; Beebee, 2005; Hansson and Westerberg, 2002; Luquet *et al.*, 2011a, 2011b; Reed and Frankham, 2003; Ribeiro and Lopes, 2013; Rowe and Beebee, 2003; Schmeller *et al.*, 2007). Furthermore, directional selection could genetically erode a population and, subsequently, if it severely downsizes the population then further genetic erosion due to genetic drift, with possible alleles' fixation, could follow (Belfiore and Anderson, 2001). This background knowledge rises an high concern regarding amphibian populations, since, this group of organisms is facing a worldwide global decline (Allentoft and O'Brien, 2010; Barinaga, Blaustein and Bancroft, 2007; Brühl *et al.*, 2013; Fedorenkova and Vonk, 2012; IUCN, 2013; McKenzie and Peterson, 2012; O'Brien and Allentoft, 2010; Puschendorf and Hodgson, 2013; Rowe and Freda, 2000; Stuart *et al.*, 2004; Wake and Vredenburg, 2008; Wind, 1999) and can be severely affected by contaminant-induced genetic erosion. Amphibian populations, impacted by chemical contamination, may undergo a process of contaminant-driven loss of genotypes and, of utmost concern, alleles. Following the recessive tolerance inheritance (working-) hypothesis (*sensu* Ribeiro and Lopes, 2013), this may happen when genetically determined tolerance (to a particular compound) is a fully recessive or incompletely dominant trait. Incomplete dominance refers to all situations where the individual phenotypic expression is not fully polymorphic (each individual being either sensitive or tolerant) but, instead, follows a gradient depending on the degree of dominance (each indiviadual being either sensitive or tolerant or in-between, from above 0% - almost fully recessive - to below 100% - almost fully dominant) of the trait (tolerance). In a previous study (Fasola *et al.*, unpublished data), incomplete dominance was found to be the likely inheritance mechanism of tolerance towards lethal concentrations of acid mine drainage in Perez's frog, if there is only a single gene involved. Acid mine drainage is a complex mixture of metals ions, so in the present paper the focus was moved towards a single metal toxicity. Furthermore, sampling and laboratory assays were performed in two different years, to investigate if the mechanism is consistent over time. Therefore, in the present study, the tolerance inheritance to copper sulphate by a natural population of Perez´s frog, *Pelophylax perezi*, was investigated. Here tolerance inheritance mechanisms were studied by: 1) investigating whether populations exhibit "critically sensitive genotypes", defined accordingly Ribeiro and Lopes

(2013) and 2) comparing among and within egg masses variability in time to death responses. If genetically determined tolerance corresponds to a recessive allele, then the most tolerant (both parents being recessive homozygous) and the most sensitive egg masses (at least one parent being dominant homozygous) should have lower variability compared with masses showing intermediate median lethal time values (LT_{50}) (both parents being heterozygous or one being recessive dominant and the other heterozygous). Indeed, the worst-case scenario of the recessive tolerance inheritance hypothesis would correspond to an inverted U-shaped relationship between the variability and the median lethal time. In this case, if exposed to a partially lethal level of the stressor, then the population would end up losing a large part of its individuals, since tolerance, being a recessive trait, would only be present in a small portion of the population. This processes match the definition of genetic erosion firstly given by Van Straalen and Timmermans (2002): the loss of genotypes determining a specific trait or set of traits. This loss of non-tolerant genotypes (regarding one specific stressor) increases the populations overall tolerance (regarding only that specific stressor). Therefore, genetic erosion reduces the pool of variation upon which natural selection can act; as a microevolutionary process it can leave natural populations deprived of the "genetic resources" to face future environmental challenges (Medina *et al.*, 2007; Ribeiro and Lopes, 2013). In the light of this theoretical framework and following the results obtained with acid mine drainage (Fasola *et al.*, unpublished data), the most likely outcome for the present work was incomplete dominance being the inheritance mechanism of the genetically determined tolerance to copper.

Material and methods

Study organism:

Pelophylax perezi López-Seoane 1885 is a frog endemic in the Iberian Peninsula and very common in rivers, streams, irrigation ditches, ponds, marshes, and reservoirs (Arnold and Ovenden, 2002; Bosch *et al*., 2013). *Pelophylax perezi* can inhabit eutrophic and polluted waters (Marques *et al*., 2013; Sillero and Ribeiro, 2010), making it a pioneer species in polluted habitats (Arnold and Ovenden, 2002).

Assay setup:

Sampling was performed in two seasons: from March 2014 to May 2014 and from March 2016 to April 2016. At each sampling season, 20 egg masses laid in the previous 24h (Gosner's stage 8 - 10) were collected, at Quinta de Boa Vista (central west coast of Portugal, 40°35’48”N – 8°41’43”W), which is considered a non-contaminated undisturbed reference site. Average data of: air temperature,

amount of rain, wind speed, and insulation for each day of egg collection, at the selected sampling site, were downloaded from the datasets of the Portuguese environmental agency (Instituto Português do Mar e da Atmosfera). The egg masses were temporarily housed in small water aquaria (20x20x30 cm) containing Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) medium (American Society for Testing and Materials International (ASTM), 2004; Organisation for Economic Co-operation and Development (OECD), 2008). The toxicity tests were performed in plastic Petri dishes (55 mm diameter) containing 10 ml test solution and 5 eggs each. The test solution was made by diluting with FETAX medium a stock solution (100 mg/L Cu) of $CuSO_4 \cdot 5H_2O$ (Merck, Kenilworth, NJ, USA), prepared with with Milli-Q water (Academic System; Merck Millipore, Darmstadt, Germany), until a 9 mg/L of Cu concentration (resulting pH≈7). This value was chosen based on concentrations tested by Santos *et al.* (Organisation for Economic Co-operation and Development (OECD), 2008) and was intended to be lethal within a 96h exposure period. Controls consisted of FETAX medium only. For each egg mass, 4 control replicates, with 5 eggs each, were used. The number of replicates exposed to copper, also with 5 eggs each, varied between 16 and 20, depending on the egg mass size. The eggs' jelly coat was not removed to mimic natural conditions. Frog embryos were placed in a climatic chamber (23°C) under a 14h/10h light/dark photoperiod, for 96 h. Embryo status, response to gentle prodding and circulatory system functioning were sequentially inspected to assess death (at 10x magnification; Leica MS5 microscope, Leica Microsystems, Wetzlar, Germany) following a logarithmic time schedule: 12h00min, 16h57min, 23h57min, 33h49min, 37h46min, 67h29min, and 95h19min. The pH (WTW pH330i, Wissenschaftlich Technische Werkstätten, Weilheim, Germany) and conductivity (WTW conductivity440i) were measured at each observation time. The assay started with embryos approximately at Gosner's stage 9-10. Control eggs reached Gosner's stage 21-22 by the end of the experiments.

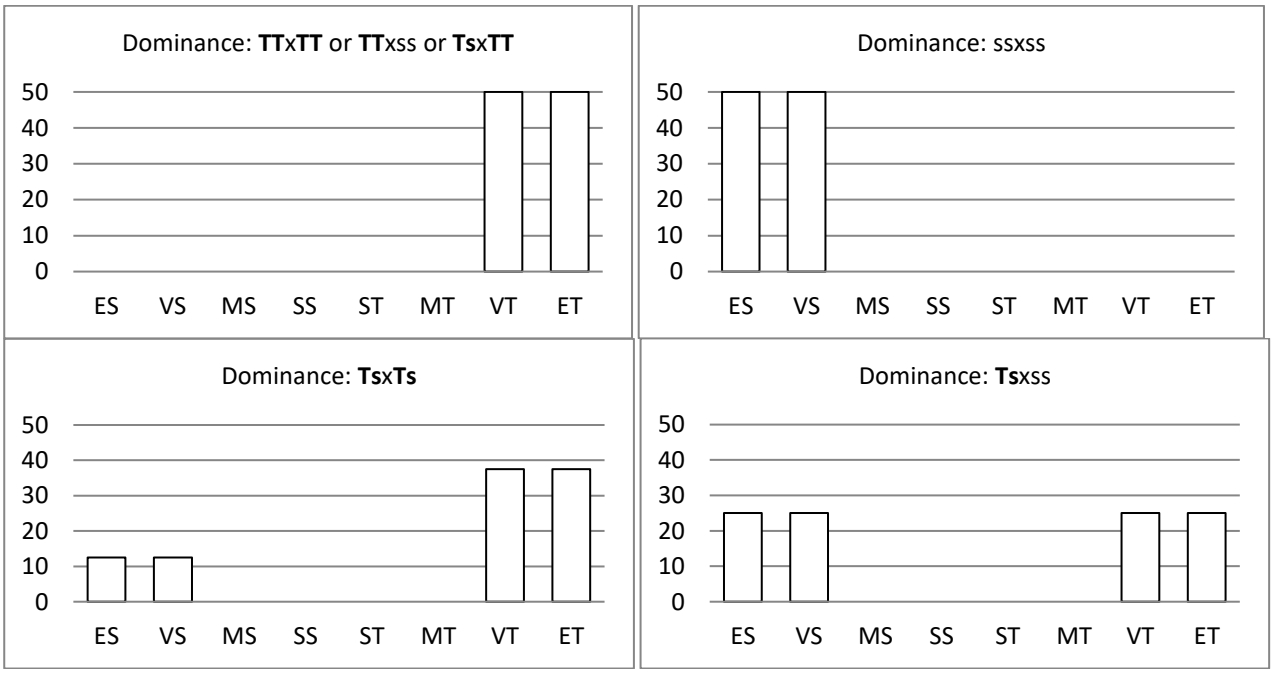
Statistical analysis:

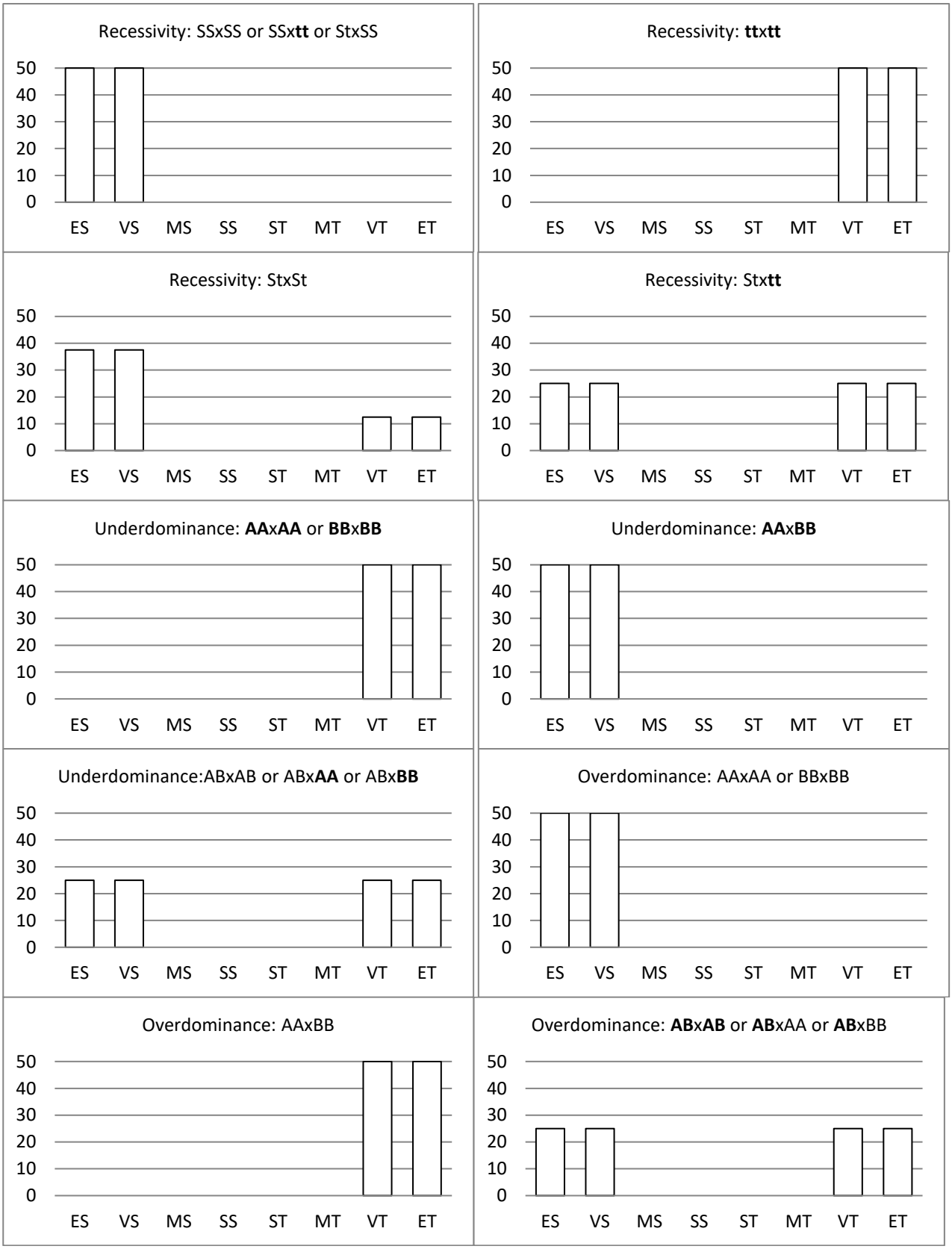
Egg masses were labelled from A14 to T14 and A16 to T16 (in order from the most sensitive to the most tolerant, i.e. increasing tolerance for each sampling season – 2014 and 2016, respectively). Each egg was classified from extremely sensitive to extremely tolerant depending on when it was found dead during the experiment. Median, lower and upper quartile of lethal time values (LT_{50}, LT_{25} and LT_{75}, respectively) were calculated, for each egg mass, using the PriProbit software (Sakuma, 1998). Egg masses were said to be critically sensitive when their LT_{75} was below the mean of the set of LT_{50} values and as safely tolerant when their LT_{25} was above the mean LT_{50} (Ribeiro and Lopes 2013). Evaluation of "within egg mass variability in time to death responses" was performed calculating their relative spread, which is the interquartile distance – LT_{75} minus LT_{25} – divided by its median –

the LT_{50} (Ribeiro and Lopes 2013). The following analyses were performed using Statistica for Windows 8.0 (StatSoft, Tulsa, OK, USA) and IBM SPSS for Windows v24 (IBM Corporation, Armonk, NY, USA). Values of LT_{50} versus their respective relative spreads were checked for parametric correlation. Investigating the possible effects of environmental parameters on tolerance, correlations of rank of the collection day, average air temperature, amount of rain, average wind speed, average insulation at each day of egg collection versus the respective LT_{50} values were explored. Comparison between LT_{50} and spread values resulting from the two sampling seasons was performed conducting a Mann-Whitney U-test.

Genetic mechanism evaluation:

To explore the genetic basis of inheritance of the tolerance mechanism, to a concentration of copper causing mortality, theoretical graphs were constructed to show the expected death pattern of F1 tolerance in the case of genetically determined tolerance being driven by a single gene with two alleles. The following outcomes were explored: tolerance being a dominant, recessive, overdominant, underdominant, or incompletely dominant trait (Fig.4.1). The degree of dominance of the tolerance trait is 50%, meaning that a heterozygotes presents a median tolerance. The eggs' sensitivity distribution of an egg mass would shift to the right or to the left if the degree of dominance differs from 50%, from almost full recessivity (dominance degree slightly above 0%) to almost full dominance (dominance degree slightly below 100%).




Recessivity: SSxSS or SSx**tt** or StxSS
Recessivity: **tt**x**tt**
Recessivity: StxSt
Recessivity: Stx**tt**
Underdominance: **AA**x**AA** or **BB**x**BB**
Underdominance: **AA**x**BB**
Underdominance:ABxAB or ABx**AA** or ABx**BB**
Overdominance: AAxAA or BBxBB
Overdominance: AAxBB
Overdominance: **AB**x**AB** or **AB**xAA or **AB**xBB
50
40
30
20
10
0
ES VS MS SS ST MT VT ET

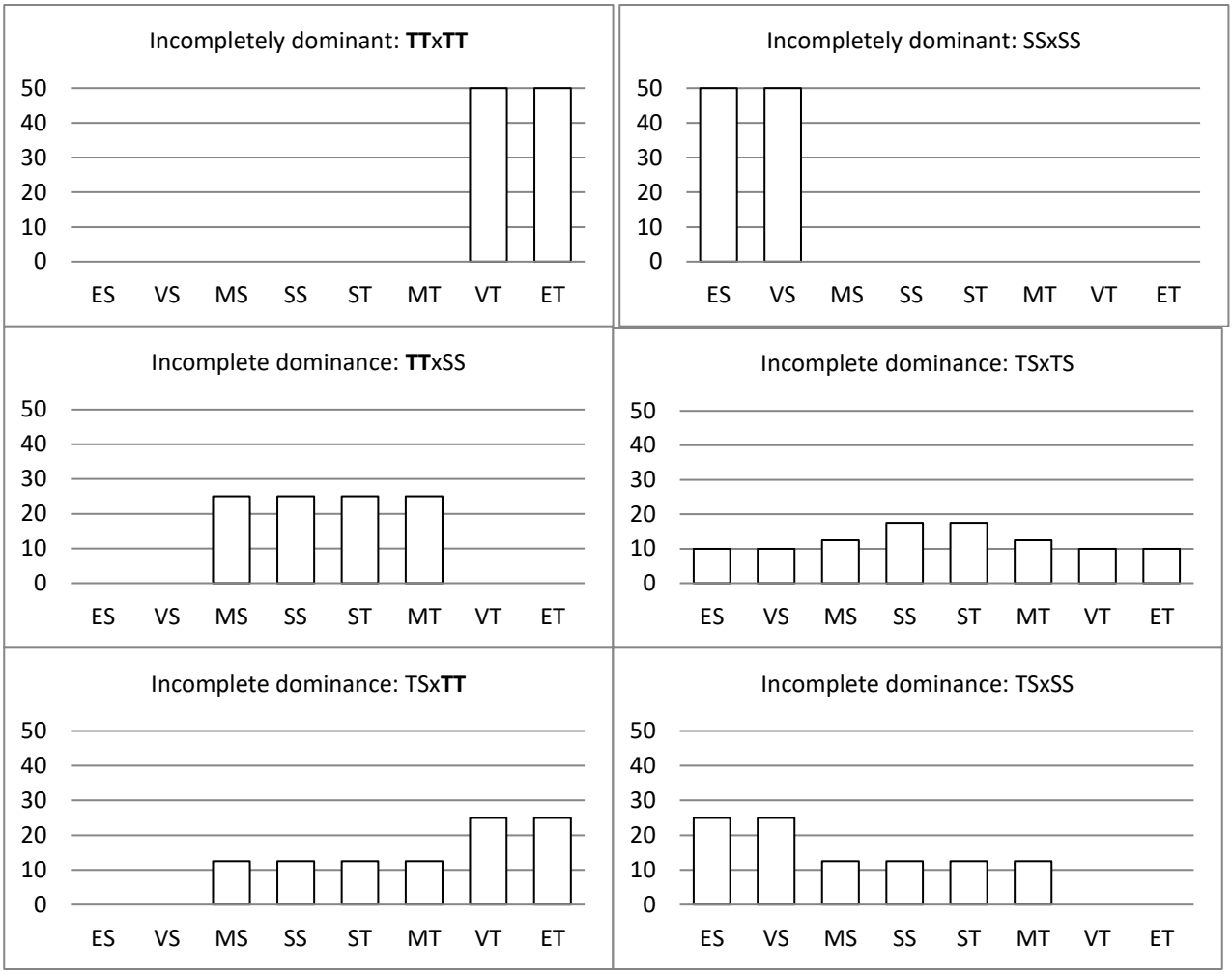


Fig.4.1: Expected theoretical frequencies (in %) of eggs (F1 generation), of Perez's frog (Pelophylax perezi) egg masses, belonging to eight classes of lethal tolerance to 9mg/L of Copper, in all possible scenarios of inheritance mechanism (dominance, recessivity, underdominance, overdominance, and incomplete dominance) from all possible parental genotype crossings in a two-alleles system (T and S) of a single gene. The classes correspond to the eight observation moments (following a logarithmic time scale from 12h00min up to 95h19min of exposure). T - allele conferring tolerance. S - allele not conferring tolerance. In the case of overdominance and underdominance, where there is not really an allele conffering tolerance, they were indicate as alleles A and B. Lower case indicates the recessive allele. Bold face indicates the most tolerant genotypes. The X-axis represents time in subsequent observations. ES, VS, MS, SS, ST, MT, VT, and ET – Extremely, Very, Moderately, and Slightly Sensitive or Tolerant eggs, respectively.

Results

All assays, performed in the present work, were considered valid since mortality in the controls never exceeded 20%. This value was reached only in 3 out of 40 egg masses. Environmental parameters, either at each collection day (rank along the breeding season, average air temperature, amount of rain, average wind speed, and average insulation at each day of egg collection), or during testing in laboratory (pH and conductivity) were not correlated with LT_{50} values ($p>0.05$). Each egg mass LT_{75} was compared with mean of the set of LT_{50}, following the concept proposed by Ribeiro and Lopes 1, to identify critically sensitive genotypes (Ribeiro and Lopes, 2013).

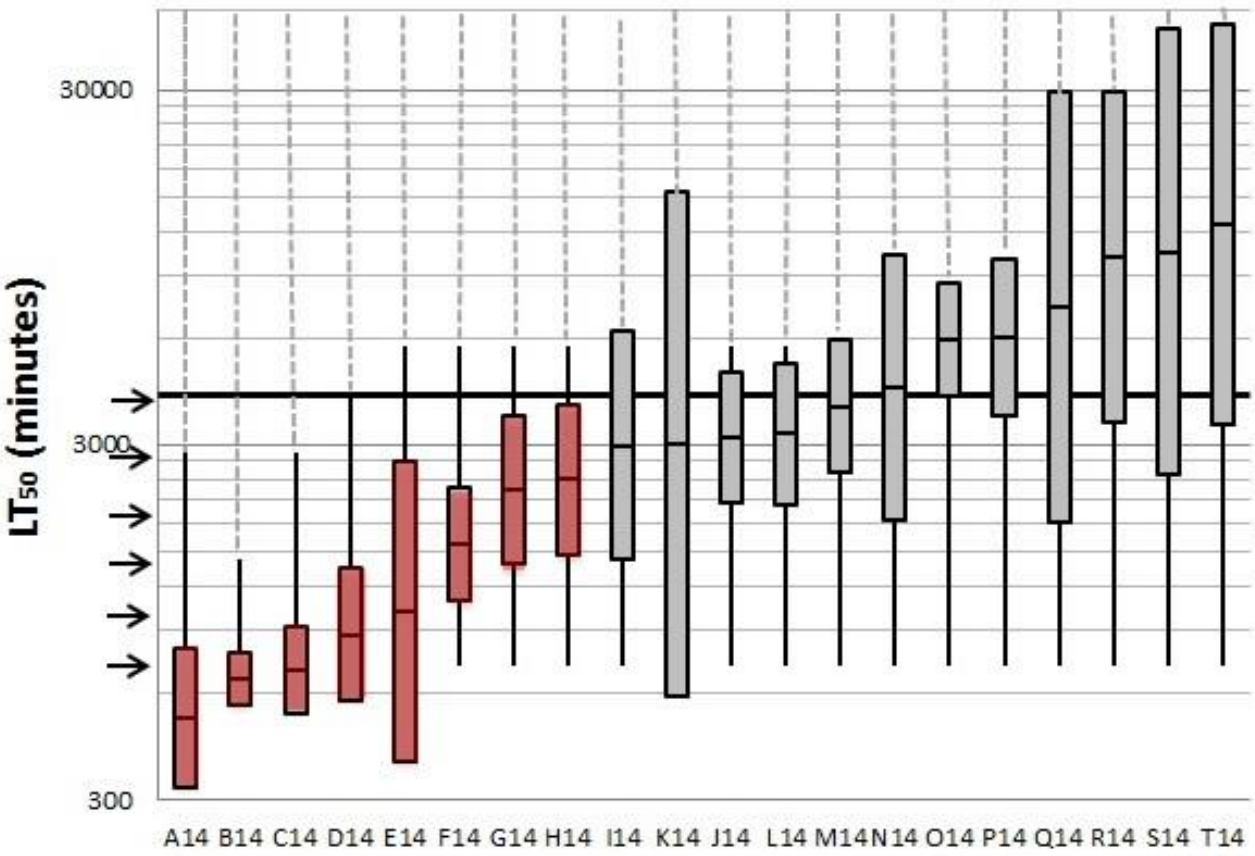
Results 2014:

The *P. perezi* sampled population presented a broad range of egg mass tolerance to copper, in 2014, with LT_{50} values covering two orders of magnitude (Table 4.1). Masses from A14 to H14 were found to be critically sensitive (having LT_{75} lower than average LT_{50}) but no safely tolerant egg masses (having LT_{25} higher than average LT_{50}) were encountered (Fig.4.2 and 4.3). Egg masses E14, I14, and K14 showed bimodal distribution patterns of egg tolerance (Fig.4.3). All egg masses had relative spread values below 300% with four exceptions: masses K14, Q14, S14 and T14 (being S14 and T14 most tolerant egg masses) (Table 4.1 and Fig.4.4).

Table.4.1: Median lethal time values (LT_{50}) with their 95% confidence limits and respective relative spread of the 20 Perez's frog egg masses exposed to copper in 2014. The average of all LT_{50} values is also indicated.

Egg Mass	LT_{50} (minutes)	LT_{50} 95% conf. limits	Rel. Spread
A14	510.32	377.34 - 613.14	94.40
B14	656.68	585.52 - 702.56	34.20
C14	697.37	617.35 - 760.68	57.60
D14	874.14	603.20 - 1080.81	88.79
E14	1018.78	805.76 - 1368.39	225.26
F14	1582.22	1339.84 -1844.95	75.19
G14	2230.76	1767.15 -2879.11	99.92
H14	2389.03	1983.21 - 2931.47	101.61
I14	2989.79	1580.31 - 4500.63	160.96
K14	3016.58	1865.46 - 4751.32	495.47
J14	3142.71	1960.32 - 8661.77	87.96
L14	3211.62	1948.36 - 10923.40	94.28
M14	3856.35	2557.67 - 10774.75	89.54
N14	4326.76	2755.45 - 17824.22	193.60
O14	5904.50	3295.35 - 19827.46	75.04
P14	6025.90	4597.20 - 10551.57	106.03
Q14	7320.20	5233.49 - 13145.48	379.90
R14	10185.10	5760.08 - 16929.11	257.35
S14	10491.40	6844.12 - 23548.46	398.92
T14	12578.20	6216.81 - 169212.03	337.74
	Mean LT_{50} (minutes)		
	4150.42		

The analysis showed a significant positive association between egg mass tolerance (LT_{50}) and the within egg mass variability (relative spread) (R=0.59; p=0.006) (Fig.4.4).



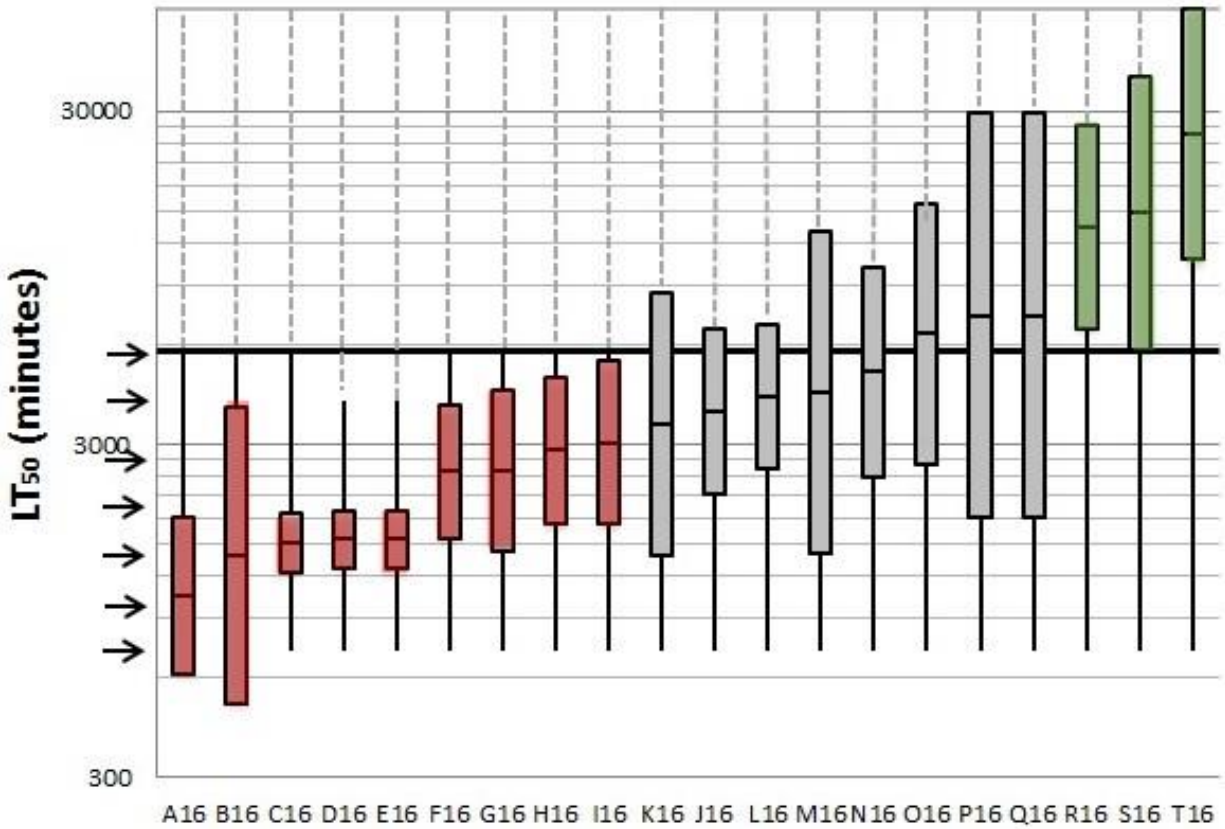
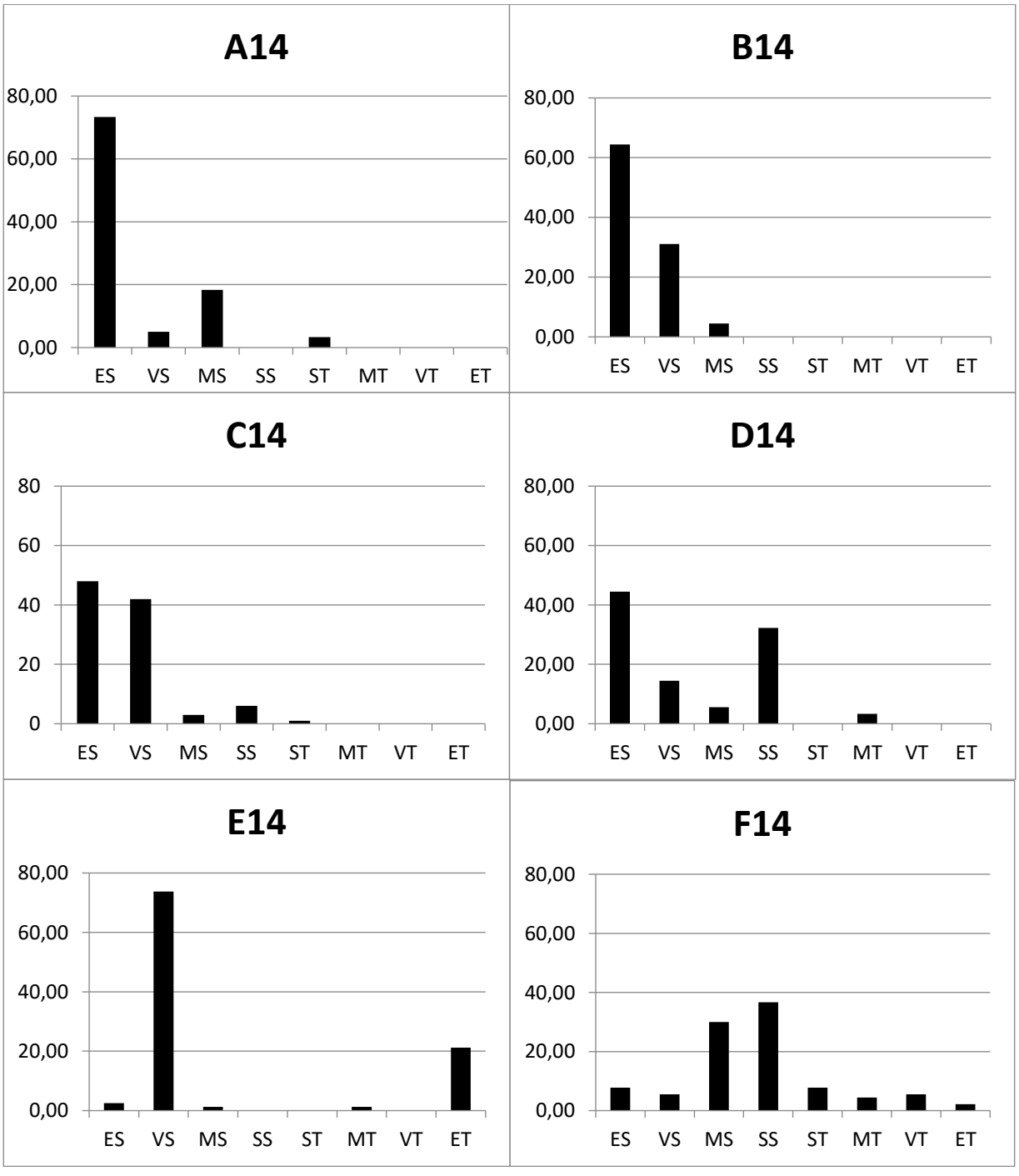
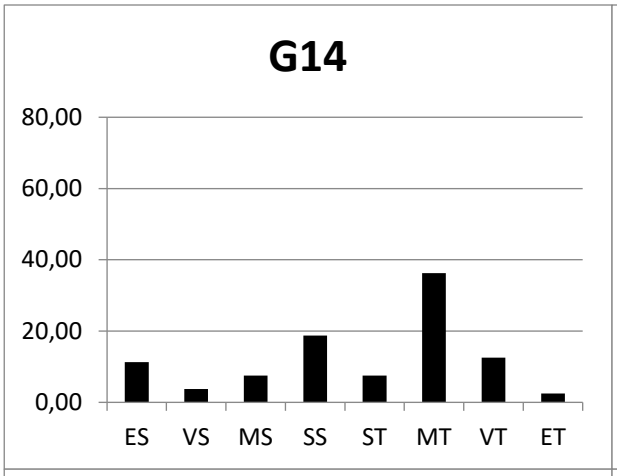
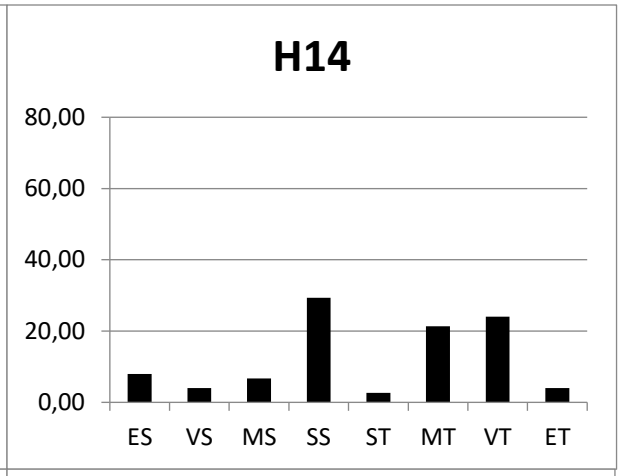


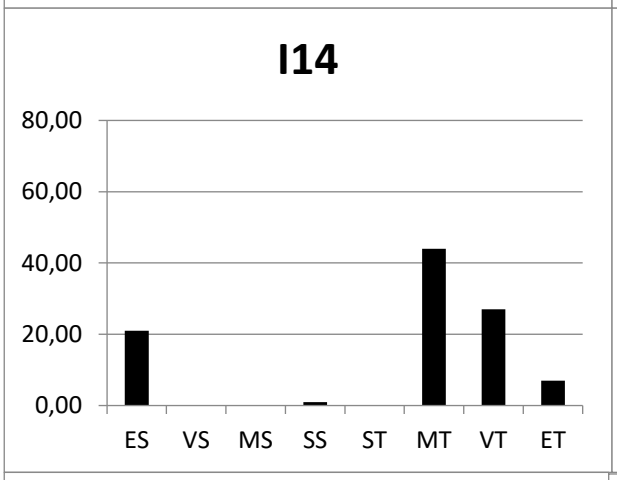
Fig.4.2: Box plots (2014 sampling above, 2016 sampling below) representing the median, the lower and the upper quartiles of lethal time values (exposure times after which 50, 25 and 75% of eggs died) of eggs within each of 20 Perez's frog (*Pelophylax perezi*) egg masses (A to T, 2014 and a to t, 2016), collected in a reference pond, exposed to 9mg/L of Copper. Vertical black lines represent maximum and minimum lethal time values for each egg mass (until the last observation made). Grey dashed lines indicate some eggs were still alive at the end of the last observation. The thick

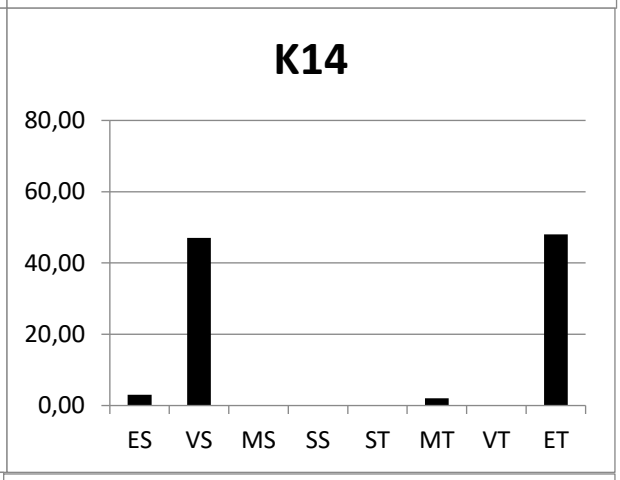
horizontal black line represents the average of the 20 median lethal time values. Observations, indicated by arrows, were made 720, 1017, 1436, 2029, 2866, 4048 and 5719 minutes after the start of the assay.

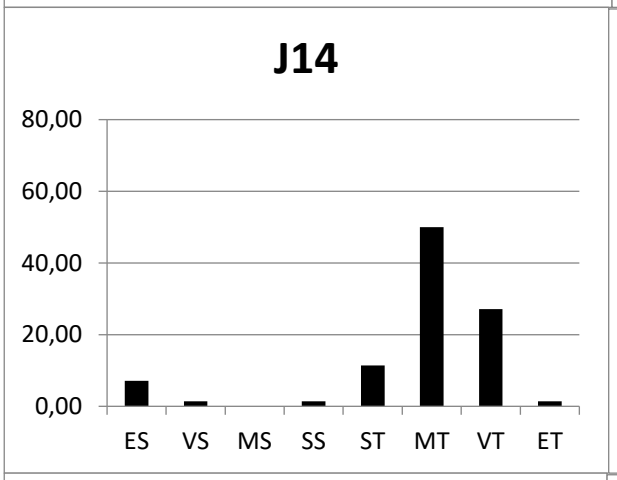


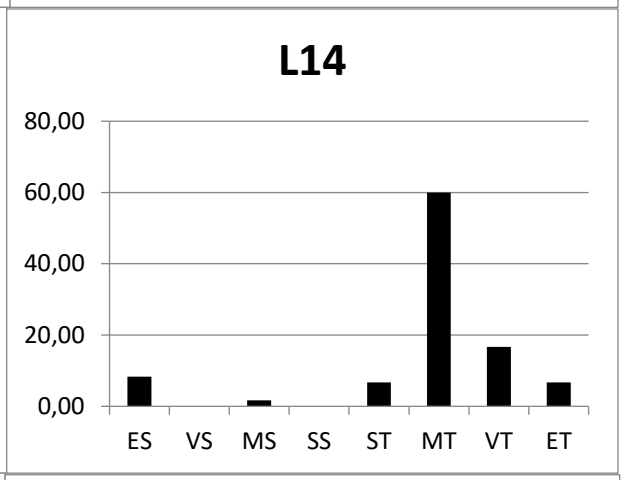

G14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET

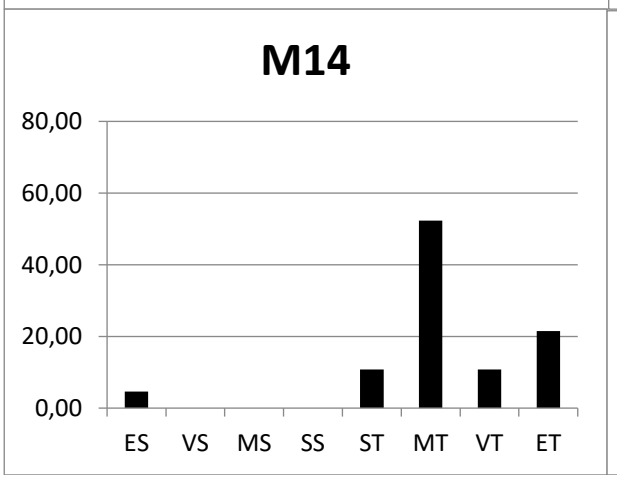

H14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET

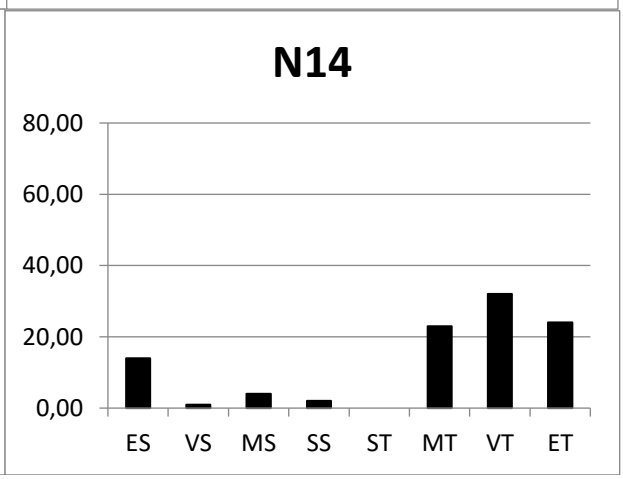

I14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET

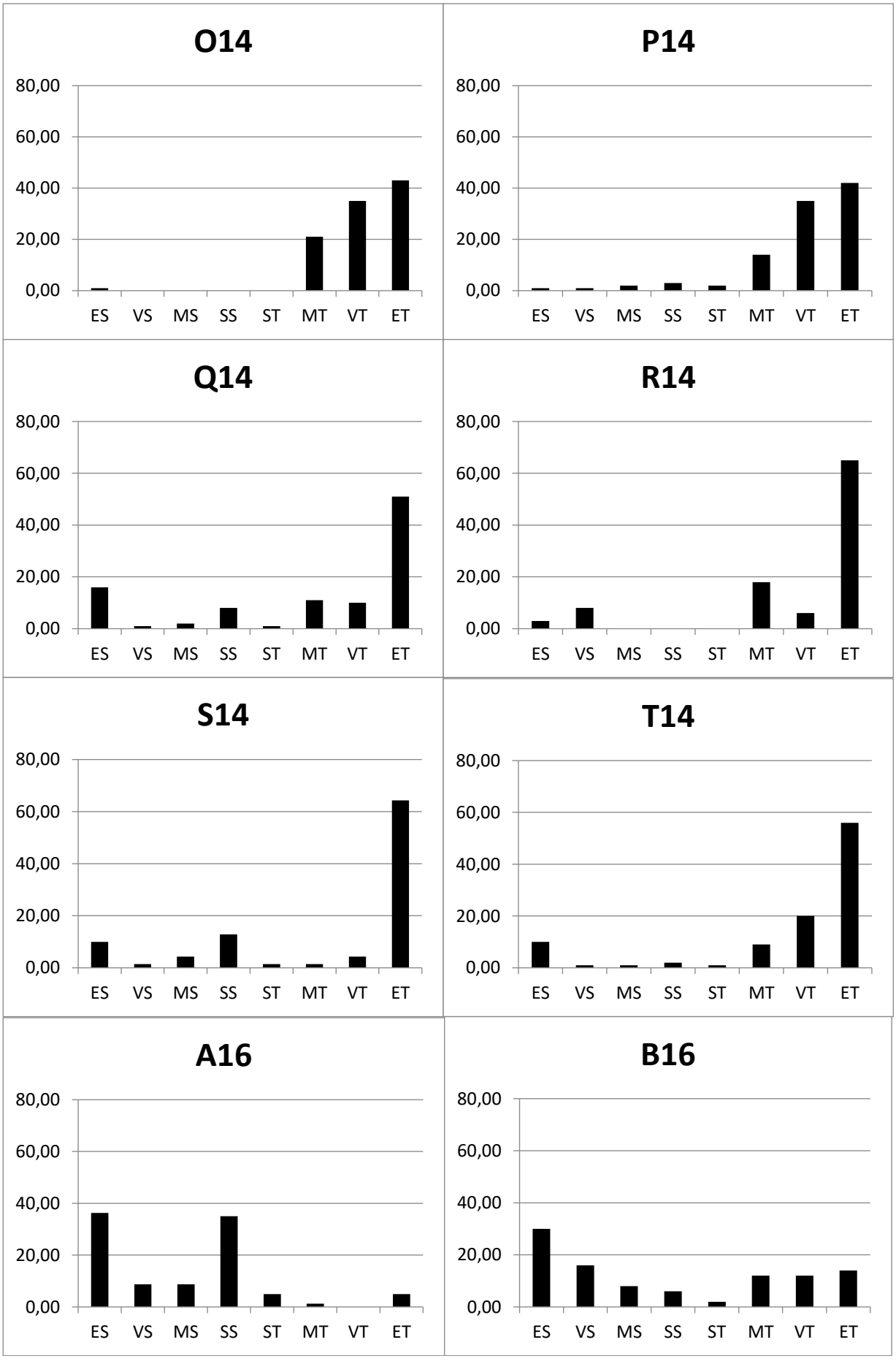

K14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET

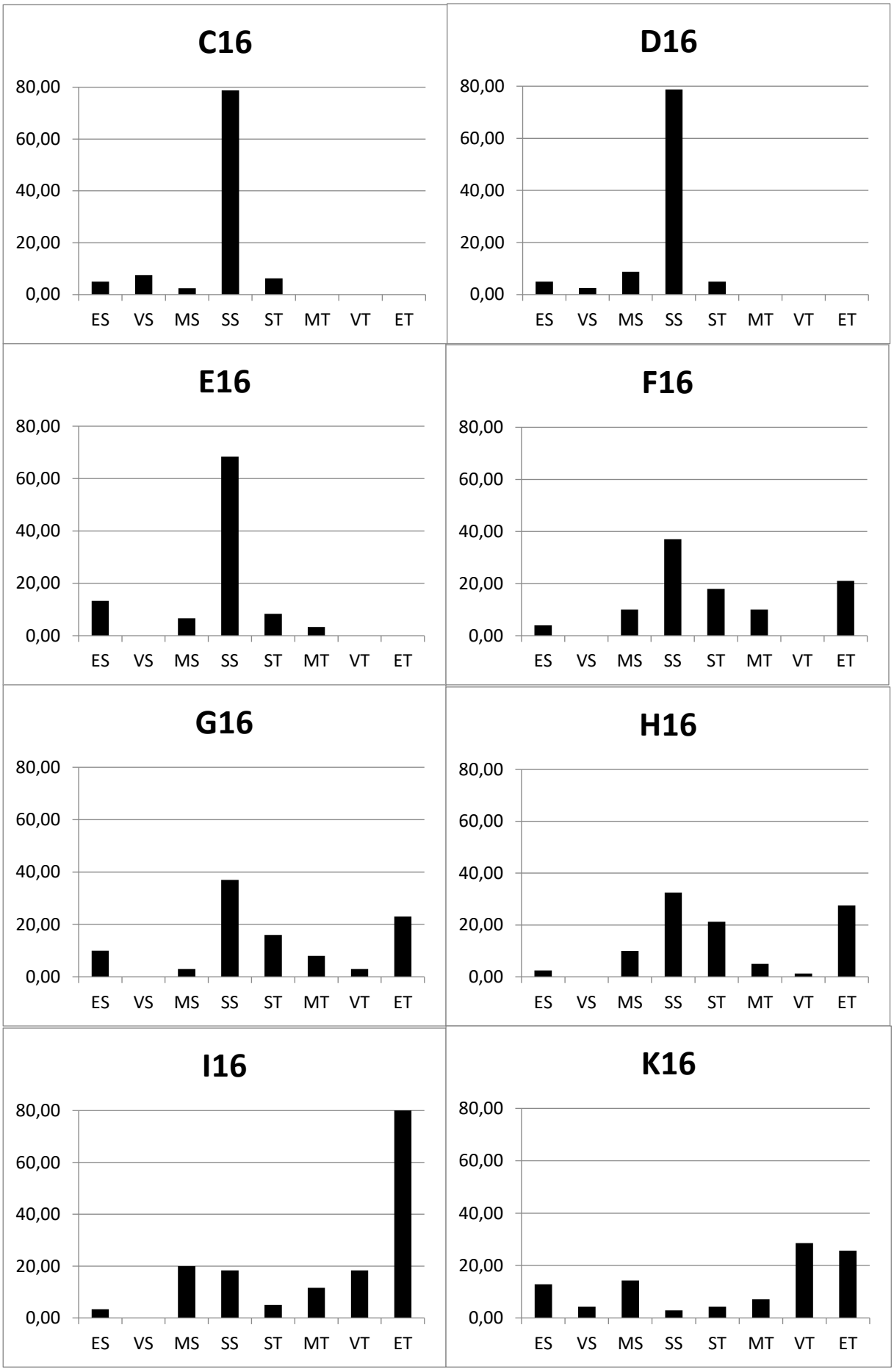

J14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET

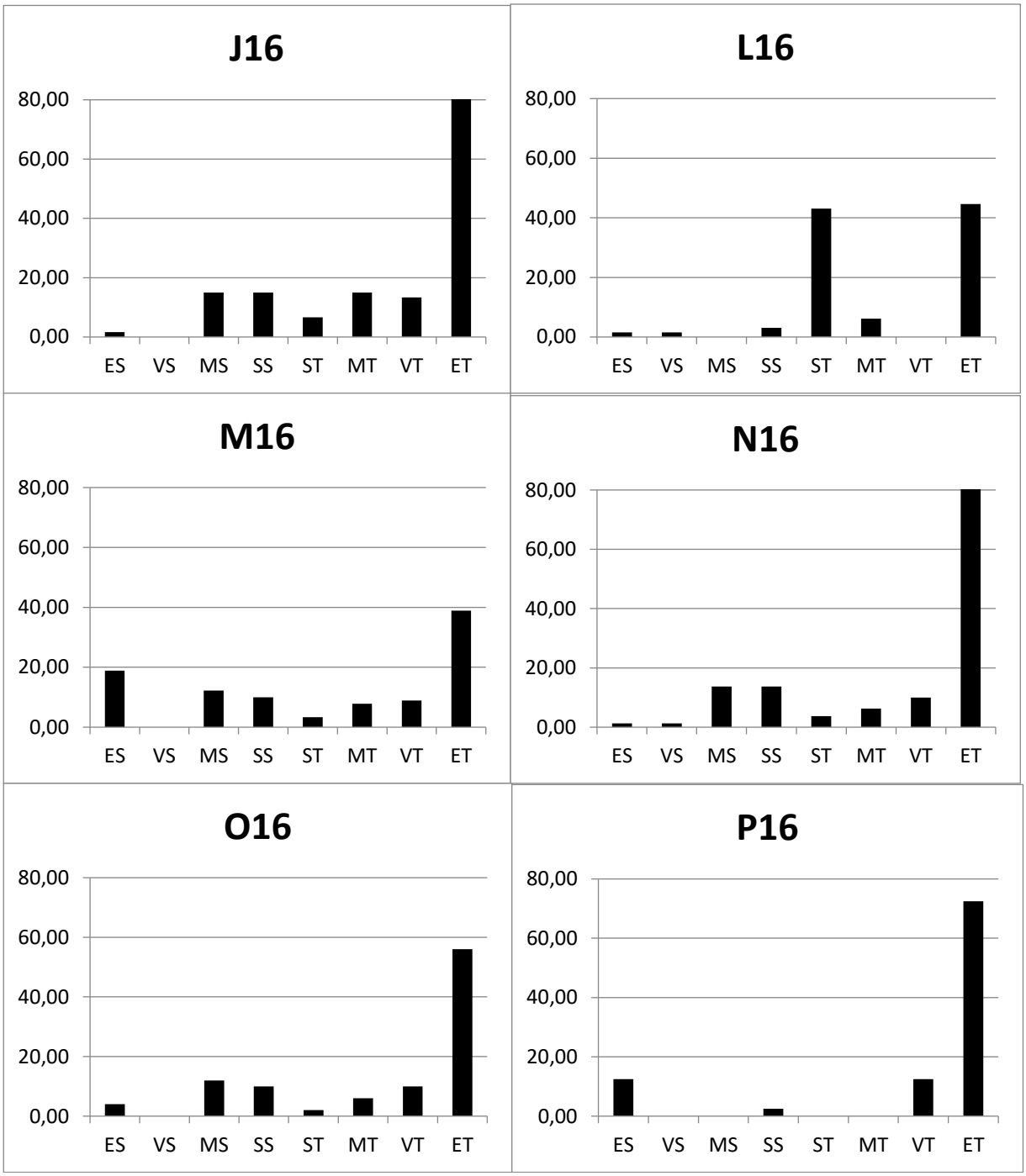

L14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET


M14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET


N14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET


O14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
P14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
Q14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
R14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
S14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
T14
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
A16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
B16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET


C16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
D16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
E16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
F16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
G16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
H16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
I16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
K16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET


J16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
L16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
M16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
N16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
O16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET
P16
80,00
60,00
40,00
20,00
0,00
ES VS MS SS ST MT VT ET

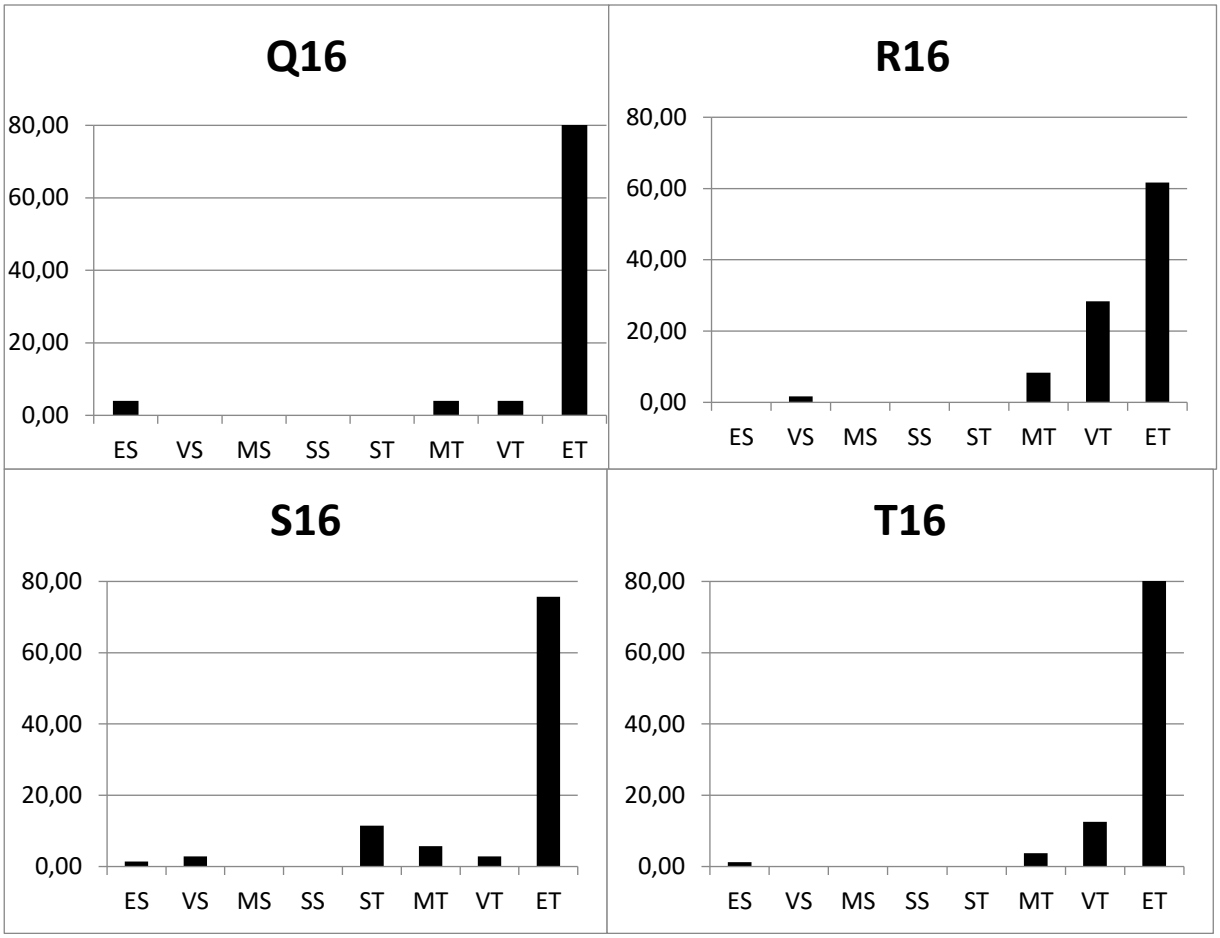


Fig.4.3: Frequencies (in %) of eggs belonging to eight classes of lethal tolerance to 9mg/L of Copper, in each of 20 Perez's frog (*Pelophylax perezi*) egg masses (A to T – 2014; a to t – 2016), collected in a reference pond. The classes correspond to the eight observation moments (following a logarithmic time scale from 12h00min up to 95h19min of exposure). The X-axis represents time in subsequent observations. ES, VS, MS, SS, ST, MT, VT, and ET – Extremely, Very, Moderately, and Slightly Sensitive or Tolerant eggs, respectively.

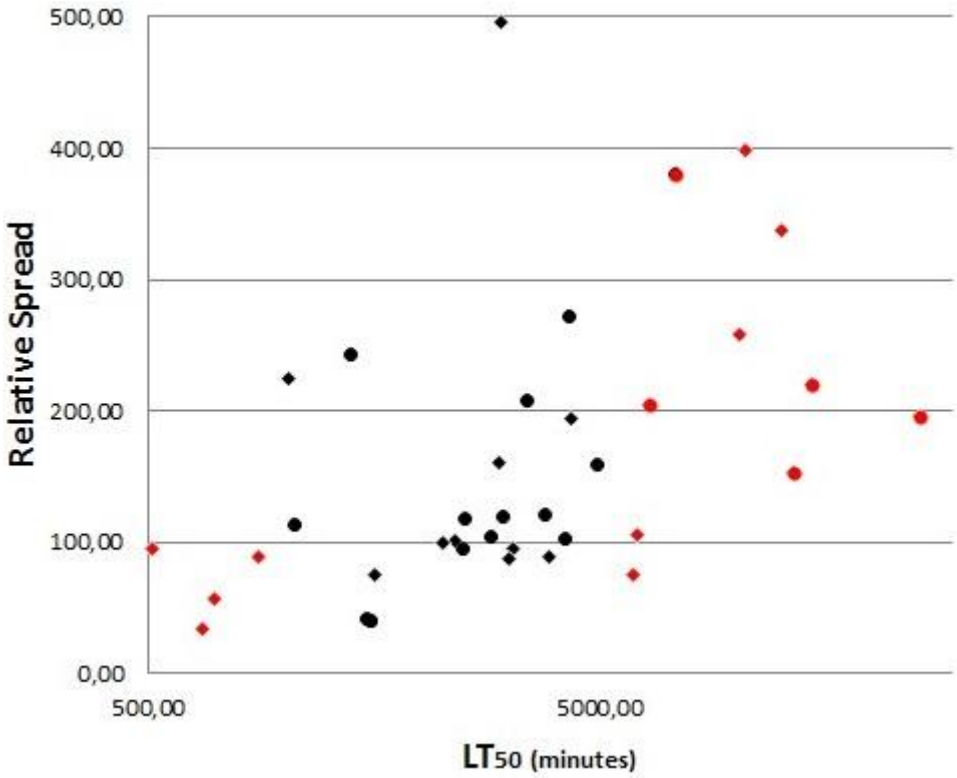


Fig.4.4: Relative spread (difference between the upper and lower quartiles relatively to the median) of egg tolerance to copper of 40 Perez's frog (*Pelophylax perezi*) egg masses (2014 egg masses are represented by diamonds and 2016 by circles), collected in a reference pond, versus the respective median lethal time values (LT_{50} – exposure time after which 50% of eggs died). Red symbols indicate when the calculated LT_{50} had a value lower than the time in which we performed the first observation (720 minutes) or higher than the last observation (5719 minutes).

Results 2016:

The observed low mortality in the controls eggs, suggests that intrinsic mortality rate, in the field (under optimal environmental conditions), of *P. perezi* egg masses is very low. Therefore, mortality in the field could be assumed to be the result of environmental stressors (for example contamination, predation or other environmental pressures). In controlled laboratory conditions high variability was recorded, between egg masses, in the tolerance to copper. These responses, following exposure to copper contamination, did not variate in function of pH or conductivity. The observed variability, in the present study, was neither function of the environmental conditions at the moment of the egg mass was laid (no correlation were found between tolerance to metal exposure and the recorded environmental variables). Therefore, it is assumed that the observed variability, in tolerance to copper, could be either: 1) stochastic or 2) genetically determined. Because of the huge variability in egg mass tolerance, the first option can be safely dismissed; thus, the observed variability can be assumed as being due to genetically determined tolerance mechanisms. The *P. perezi* population, at the sampling site, showed a broad range of egg mass tolerance to copper also in 2016, with LT_{50} values covering two orders of magnitude (Table 4.2). Masses from A16 to I16 were found to be critically

sensitive (having LT_{75} lower than average LT_{50}) and masses from R16 to T16 were identified as safely tolerant (having LT_{25} higher than average LT_{50}) (Fig.4.2 and 4.3). Only egg mass K16 showed a bimodal distribution of egg tolerance (Fig.4.3). All egg masses had relative spread values below 300% with two exceptions: masses P16 and Q16 (Table 4.2 and Fig.4.4).

Table 4.2: Median lethal time values (LT_{50}) with their 95% confidence limits and respective relative spread of the 20 Perez's frog egg masses exposed to copper in 2016. The average of all LT_{50} values is also indicated.

Egg Mass	LT_{50} (minutes)	LT_{50} 95% conf. limits	Rel. Spread
A16	1045.72	696.59 - 1337.77	114.03
B16	1393.14	1157.02 - 1622.63	242.89
C16	1525.32	1143.42 - 2151.83	41.52
D16	1553.49	1273.12 - 2352.80	40.39
E16	1555.91	1279.27 - 2452.87	40.39
F16	2477.99	1933.65 - 3249.81	95.48
G16	2496.92	1944.36 - 3334.01	118.45
H16	2868.43	2201.98 - 4001.46	104.40
I16	3035.57	2526.89 - 3814.64	118.92
K16	3454.40	1865.46 - 4751.32	208.13
J16	3761.61	2981.14 - 4157.42	121,64
L16	4180.08	3087.37 - 7651.67	103.20
M16	4272.60	3505.26 - 5654.01	271.42
N16	4940.60	3683.04 - 8593.13	159.15
O16	6434.97	5168.01 - 8887.32	204.90
P16	7320.20	5233.49 - 13145.40	379.90
Q16	7321.29	5239.90 - 13148.48	379.90
R16	13483.70	6700.64 - 32251.20	153.02
S16	14798.90	9717.50 -31683.00	219.93
T16	25515.50	13703.41 -106548.21	195.31
	Mean LT_{50} (minutes)		
	5671.64		

The analysis showed no significant association between egg mass tolerance (LT_{50}) and the within egg mass variability (relative spread) ($p>0.05$) and no clear pattern was observed (Fig.4.4).

Comparison between 2014 and 2016 sampling seasons:

The LT_{50} and relative spread values recorded in 2016 were slightly higher than those of 2014, but the differences between the two sampling seasons were not significant ($p>0.05$) (Tables 1 and 2). Three safely tolerant masses were found in the 2016 sampling year, while none was registered in 2014. Within each egg mass, a bimodal pattern of egg tolerance was found in 4 out of 20 egg masses in 2014, and only 1 out of 20 in the 2016 sampling season.

Comparison between AMD and copper tolerance:

Comparing the results from the two years of sampling in the present study with those of the previous study on acid mine drainage (AMD) (Fasola *et al*., unpublished data), copper showed median lethal time values higher than those recorded for AMD (average LT_{50} for copper in 2014 = 4150,42 min, average LT_{50} for copper in 2016 = 5671,64 min, average LT_{50} for AMD = 1966 min), though LT_{50} values for copper were not statistically different than those of AMD both for 2014 and 2016 ($p>0.05$) (Fig.5). The same is valid for relative spread values ($p>0.05$). Three safely tolerant masses (all in 2016) and 17 critically sensitive egg masses out of 40 were found in the copper study, while 2 and 10 out of 21, respectively, were found in the AMD study, revealing a high degree of similarity. A bimodal pattern of egg tolerance was found in 5 out of 40 egg masses, exposed to copper, and only 1 out of 21 egg masses exposed to AMD. A significant association between LT_{50} values and spread values was not recorded in the AMD study (Fasola *et al*., unpublished data), while it was present in the 2014 sampling season, but not in the 2016, for the copper study.

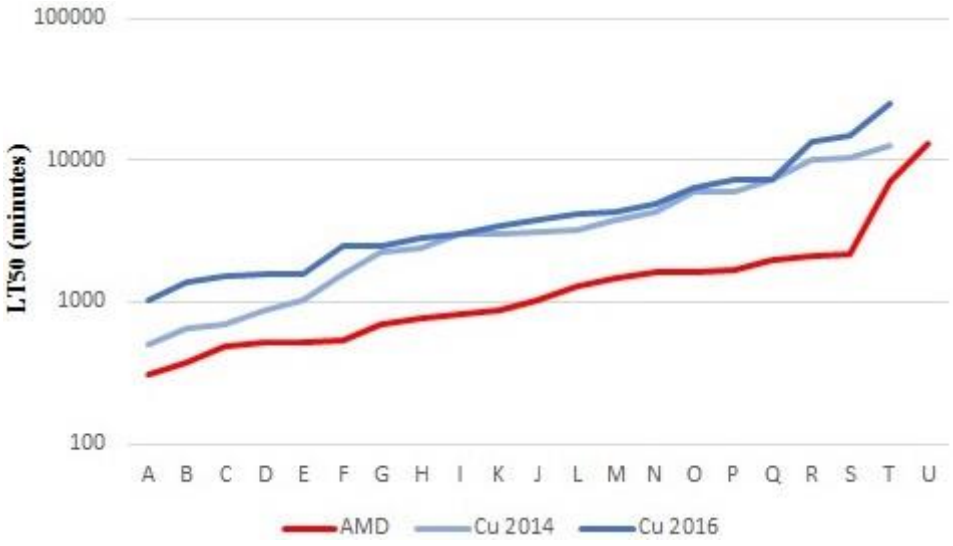


Fig.4.5: Comparison of egg tolerance (median lethal time values, LT_{50}) of Perez's frog (*Pelophylax perezi*) twenty one egg masses exposed to a dilution of metal-rich acid mine drainage (red line) and to copper (twenty masses in 2014, light blue line; twenty masses in 2016 dark blue line).

Discussion and conclusions

In this work, median lethal time values showed neither a trend during the breeding season, nor correlations with any environmental parameter at the sampling site. Thus, the obtained results were most probably due to genetic determination and, at a much lesser extent, to stochasticity.

In the present study, some egg masses with a relative spread much larger than all other were found (Tables 4.1 and 4.2, Fig.4.3). When an egg mass showed such a large relative spread, recorded for

masses K14, Q14, S14, T14, and K16, it resulted from the simultaneous presence of extremely tolerant and extremely sensitive eggs and the almost or full absence of intermediately tolerant eggs (Fig.4.3). This U-shaped bimodal distribution of egg tolerance, with only a 12.5% occurrence, could be determined by tolerance being a trait other than incompletely dominant (Fig.4.3) or arise from a polyandric mating (Lodé and Lesbarrères, 2004). However, because attendance of multiple males at the same fertilization event is rare for *P. perezi* (Arnold and Ovenden, 2002), it is most likely that only one male fertilized each egg mass.

The recessive tolerance inheritance (working-) hypothesis identifies (full) recessivity as its worst-case scenario (Ribeiro and Lopes, 2013). In this case, a partially lethal pulse of copper contamination would eradicate all the most sensitive genotypes, from the exposed population. In this situation the recessive allele would be removed. For the worst-case scenario to occur, the sampled egg masses should fit in one the following categories: (i) all eggs being sensitive, with a small relative spread (approximately matched by egg masses A14, B14, C14, C16, D16, and E16), resulting from at least one parent being homozygous dominant; (ii) all eggs being tolerant (also with a small relative spread; no sampled egg mass could ascribed to this category), resulting from both parents being homozygous recessive; or (iii) eggs being either tolerant or sensitive with a large relative spread (matched by egg masses with bimodal distribution, K14, Q14, S14, T14, and K16), resulting from the crossing of a heterozygous with either a homozygous recessive or another heterozygous (Fig.4.3). In the present study, 11 out of 40 egg masses (27.5%) were found to possibly support (full) recessivity as a mechanism for tolerance to copper in *P. perezi* tadpoles (Fig.4.3).

Furthermore, under this scenario of (full) recessivity, relative spreads of intermediately tolerant egg masses should have been larger than spread values recorded for both sensitive and tolerant egg masses; this would result in an inverted U-shaped association of relative spread versus respective tolerance. This relationship was not found in the present study (Fig.4.4). A significant association between LT_{50} values and spread values was found in the 2014 sampling season, but not in the 2016, meaning that egg mass tolerance and the within egg mass variability were positively correlate in 2014. This result was not consistent, in the same population, for the 2016 season. The observed outcome could be due to the higher occurrence of bimodality in 2014 masses, compared to those sampled in 2016.

The recessive tolerance inheritance (working-) hypothesis also identifies a second scenario for genetically determined tolerance inheritance: incomplete dominance (Ribeiro and Lopes, 2013). Incomplete dominance was supported by many of the sampled eggs masses sampled in the present work (up to 35 out of 40 patterns could be explained by incomplete dominance; also depending on the degree of dominance - almost full recessivity to almost full dominance). Furthermore, most of the

egg masses (26 out of 40) showed broad ranges of egg tolerance, intermediate relative spread values and a unimodal distribution, explainable only by incomplete dominance (Fig.4.3). Thus, genetically determined tolerance to copper could be supported as an incomplete dominant trait by at least 65% and up to 87.5% of the collected egg masses. In this context, a partially lethal pulse of copper would wipe out the most sensitive genotypes, but would not result in the fixation of the allele responsible for the tolerance to copper. Allele's fixation would happen only in the case of exposure to an almost fully lethal concentration of copper (only the homozygous tolerant genotypes would survive), depending on the degree of dominance shifting between almost full recessivity to almost full dominance.

The last possible situation is that of tolerance being a (fully) dominant, overdominant or underdominant trait. In this case, not even an almost fully lethal input of copper would eradicate alleles from the exposed population. Under this scenario, the heterozygote would be maximally tolerant (dominance and overdominance) or both homozygous would be the most tolerant individuals (underdominance). In this study, 8 (B14, E14, I14, K14, O14, R14, R16, and T16), out of 40 egg masses (20%) could match this situation (Fig.4.1 and 4.3).

Tolerance to copper, in Perez's frog tadpoles, was most likely be due to an incomplete dominance mechanism of genetically determined tolerance; which could be matched by the characteristics of most of the collected egg masses. However, neither of the alternative scenarios, discussed above, can totally be excluded, because they could be supported by at least 20% of the egg masses. In the present study, these deductions were drawn according to a genetic system of a single gene. This is a likely assumption because previous studies reported several examples of a few major genes determining the tolerance selective pressure was very intense (Hoffmann & Parsons 1991; Macnair 1997; Parsons 1997). Other systems of genetic determination could be possible, for example a situation in which more than two alleles at a single gene would produce a gradient of tolerance, but incomplete dominance would still be the most probable mechanism; because this scenario is the one supported by the largest percentage (87.5%) among the tested egg masses.

The present study results match the those of the study previously conducted with acid mine drainage (Fasola *et al*., unpublished data), when comparing the obtained safely tolerant egg masses (R16, S16 and T16, only 3 out of 40) with those critically sensitive (17 out of 40), in the sampled frog population. A lack of past selection for tolerance to copper, in the sampled population should explain this outcome. A comparison between exposure to copper and AMD would be that resulting by the respective obtained values of median lethal time. Even if not significant, there is a tendency for the latter being lower than the former (for both sampling seasons) (Fig.4.5). If this is the case then, tolerance to acid mine drainage may involve different physiological mechanisms than that to copper

alone. An explanation could be found in the different pH of the effluent and the copper solution, the former being notably acid while the latter rather neutral. Indeed, pH can influence the mortality caused by metal ions as stated by the biotic-ligand-model theory (Di Toro *et al.*, 2001). This model showed how, at least for fish (but arguably for others aquatic vertebrates breathing through gills), increasing pH decrease copper ions absorption (Di Toro *et al.*, 2001). However, because the acid mine drainage contains much more than copper alone (Sobral *et al.*, 2013), its toxicity is possible due also to other ions or the complex interaction between them.

In the defined context, incomplete dominance is supported as the most probable inheritance mechanism of tolerance to copper (87,5% of the cases). However, it is not possible to totally exclude dominance, recessivity, underdominance, or overdominance (Fig.4.1), because some egg masses (12.5%) show bimodal patterns which could not be explained by incomplete dominance and which could suggest poligeny. This makes sense in the light of the above mentioned difference in the obtained values of median lethal time, between AMD and copper, being the former lower than the latter. When a contaminant impact is milder (copper), tolerance tends to have a polygenic basis, possibly involving many minor genes, while when a contaminant causes higher lethality (AMD), few genes are probably responsible for the tolerance (Hoffmann and Parsons, 1991; Macnair, 1991, 1997).

In conclusion, the results of the present study, are in line with the results obtained in the previous assays with AMD (Fasola *et al.*, unpublished data); indicating incomplete dominance as the most probable mechanism of inheritance of genetically determined tolerance to metal contamination if a single gene is involved.

References

ADLASSNIG, Wolfram; SASSMAN, Stefan; GRAWUNDER, Anja; PUSCHENREITER, Markus; HORVATH, Amadea; KOLLER-PEROUTKA, Marianne - Amphibians in metal-contaminated habitats. **Salamandra**. 49:3 (2013) 149–158.

ALLENDORF, F. W.; LEARY, R. - Heterozygosity and fitness in natural populations of animals. In Conservation biology: the science of scarcity and diversity. [S.l.] Soule M.E. (ed). **Conservation Biology**. Sinauer: Sunderland, MA, (1986) p. 75–76.

ALLENTOFT, ME; O'BRIEN, J. - Global amphibian declines, loss of genetic diversity and fitness: a review. **Diversity**. 2010).

AMERICAN SOCIETY FOR TESTING AND MATERIALS INTERNATIONAL (ASTM) - Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX) (2004) 1439–98.

ARNOLD, Nicolas; OVENDEN, Denys - Reptiles and Amphibians of Britain and Europe. [S.l.] : Collins; New edition, (2002).

BARATA, Carlos; BAIRD, Donald J.; MARKICH, Scott J. - Influence of genetic and environmental factors on the tolerance of *Daphnia magna* Straus to essential and non-essential metals. **Aquatic Toxicology**. ISSN 0166445X. 42:2 (1998) 115–137. doi: 10.1016/S0166-445X(98)00039-3.

BARINAGA, M. - Where have all the froggies gone? **Science**. 2 (1990) 1033–1034.

BEEBEE, T. J. C. - Conservation genetics of amphibians. **Heredity**. ISSN 0018-067X. 95:6 (2005) 423–7. doi: 10.1038/sj.hdy.6800736.

BELFIORE, Natalia M.; ANDERSON, Susan L. - Effects of contaminants on genetic patterns in aquatic organisms: A review. **Mutation Research**. ISSN 13835742. 489:2–3 (2001) 97–122. doi: 10.1016/S1383-5742(01)00065-5.

BLAUSTEIN, AR; BANCROFT, BA - Amphibian population declines: evolutionary considerations. **BioScience**. 57:5 (2007).

BOSCH, J.; TEJEDO, M.; BEJA, P.; MARTINEZ-SOLANO, I.; SALVADOR, A.; GARCIA-PARIS, M.; RECUERO Gil, E.; BEEBEE, T.J.C. - *Pelophylax perezi*. **The IUCN Red List of Threatened Species.** [On line], available at: http://dx.doi.org/10.2305/IUCN.UK.2009.RLTS.T58692A11812894.en.

BRÜHL, C; SCHMIDT, T; PIEPER, S; ALSCHER, A - Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? **Scientific reports** (2013) 1–4. doi: 10.1038/srep01135.

CBD - Convention on Biological Diversity [On line], available at: www.cbd.int/.

DI TORO, D. M. et al. - Biotic ligand model of the acute toxicity of metals. 1. Technical basis. **Environmental toxicology and chemistry/SETAC.** ISSN 07307268. 20:10 (2001) 2383–2396. doi: 10.1897/1551-5028(2001)020<2383:blmota>2.0.co;2.

FASOLA, E.; RIBEIRO, R.; LOPES, I. - Microevolution due to pollution in amphibians: A review on the genetic erosion hypothesis. **Environmental Pollution**. ISSN 02697491. 204 (2015) 181–190. doi: 10.1016/j.envpol.2015.04.027.

FEDORENKOVA, A.; VONK, JA - Ranking ecological risks of multiple chemical stressors on amphibians. **Environmental Toxicology and Chemistry**. 31:6 (2012) 1416–1421. doi: 10.1002/etc.1831.

GAHAN, L.; GOULD, F.; HECKEL, D. G. - Identification of a gene associated with Bt resistance in *Heliothis virescens*. **Science**. 293 (2001) 857–860.

GREAVES, J. H.; RENNISON, B. D.; REDFERN, R. - Resistance of the ship rat, *Rattus rattus* L., to warfarin. **Journal of stored products**. 12 (1976) 65–70.

HANSSON, B.; WESTERBERG, L. - On the correlation between heterozygosity and fitness in natural populations. **Molecular Ecology** (2002).

HARIDI, A. M. - Linkage studies on DDT and dieldrin resistance in species A and species B of the *Anopheles gambiae* complex. **Bulletin of the World Health Organization**. ISSN 00429686. 50:5 (1974) 441–448.

HOFFMANN, A. A.; PARSONS, P. A. - Evolutionary genetics and environmental stress. Oxford, UK ed. [S.l.] : Oxford University Press, 1991

HOFFMAN A.; WILLI, Y. - Detecting genetic responses to environmental change. Nat. Rev. Genet. 9 (2008) 431–432.

IUCN - IUCN [On line], available at: www.iucn.org/.

JAGADEESAN, R.; NAYAK, M.K.; EBERT, P.R. - Inheritance of resistance to phosphine in the rusty grain beetle,

Cryptolestes ferrugineus (Stephens). **In 11th International Working Conference on Stored Product Protection** (2014), 453-460.

KNASMÜLLER, S; GOTTMAN, E; GOD, R; KUNDI, M - Detection of genotoxic effects of heavy metal contaminated soils with plant bioassays. **Mutation research**. ISSN 0027-5107. 420:1–3 (1998) 37–48. doi: 10.1016/S1383-5718(98)00145-4.

LINDER, G.; GRILLITSCH, B. - Ecotoxicology of metals. Ecotoxicology of Amphibians and Reptiles. SETAC (2000).

LODÉ, T.; LESBARRÈRES, D. - Multiple paternity in *Rana dalmatina*, a monogamous territorial breeding anuran. **Naturwissenschaften**. 91:1 (2004) 44–47. doi: 10.1007/s00114-003-0491-7.

LOVELL, B. J.; KEARNS, C. W. - Inheritance of DDT-Dehydrochlorinase in the House Fly. **Juornal of economic enthomology** (1959) 931–935. doi: http://dx.doi.org/10.1093/jee/52.5.931.

LU, Xiang-yun; ZHANG, Yan-pin; WU, Hai-dong; ZHAO, Xiang - Study on Joint Toxicity of Mercury ion and Copper ion to *Bufo gargarizans* Tadpole. **Sichuan Journal of Zoology**. 2 (2006).

LUQUET, E. LENA, J.; DAVID, P.; JOLY, P.; LENGAGNE, T.; PERRIN, N.; PLENET, S. - Consequences of genetic erosion on fitness and phenotypic plasticity in European tree frog populations (*Hyla arborea*). **Journal of evolutionary biology**. ISSN 1420-9101. 24:1 (2011) 99–110. doi: 10.1111/j.1420-9101.2010.02138.x.

LUQUET, E; DAVID, P; LENA, J; JOLY, P; KONECNY, L; DUFRESNES, C; PERRIN, N; PLENET, S - Heterozygosity-fitness correlations among wild populations of European tree frog (*Hyla arborea*) detect fixation load. **Molecular ecology.** ISSN 1365-294X. 20:9 (2011) 1877–1887. doi: 10.1111/j.1365-294X.2011.05061.x.

MACNAIR, M. N. - The genetics of metal tolerance in natural populations. In SHAW, J. (Ed.) - Heavy metal tolerance in plants: evolutionary aspects. CRC press. ed. p. 235–254.

MACNAIR, M. N. - Why the evolution of resistance to anthropogenic toxins normally involves major gene changes: The limits to natural selection. **Genetica**. 84 (1991) 213–219.

MACNAIR, M. N. - The evolution of plants in metal-contaminated environments. In VERLAG, BIRKHAUSER (Ed.) - Environmental Stress. Adaptation and Evolution, by Bijlsma R. and Loeschcke V. Basel, Switzerland : [s.n.]. p. 3–24.

MARQUES, Sérgio; CHAVES, Sandra; GONÇALVES, Fernando; PEREIRA, Ruth - Differential gene expression in Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine. **Ecotoxicology and environmental safety**. ISSN 1090-2414. 87 (2013) 115–9. doi: 10.1016/j.ecoenv.2012.10.010.

MCKENZIE, VJ; PETERSON, AC - Pathogen pollution and the emergence of a deadly amphibian pathogen. **Molecular ecology** (2012) 5151–5154.

MEDINA, MH; CORREA, JA; BARATA, C - Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. **Chemosphere**. 67 (2007) 2105–2114. doi: 10.1016/j.chemosphere.2006.12.024.

MUSSALI-GALANTE, P. *et al*. - Evidence of population genetic effects in *Peromyscus melanophrys* chronically exposed to mine tailings in Morelos, Mexico. **Environ Sci Pollut Res Int**. 20:11 (2013) 679–766.

NADAL, M.; SCHUHMACHER, M.; DOMINGO, J. L. - Metal pollution of soils and vegetation in an area with petrochemical industry. **Science of the Total Environment**. ISSN 00489697. 321:1–3 (2004) 59–69. doi: 10.1016/j.scitotenv.2003.08.029.

O'BRIEN, John; ALLENTOFT, Morten E. - Global Amphibian Declines, Loss of Genetic Diversity and Fitness: A Review. **Diversity**. ISSN 1424-2818. 2:1 (2010) 47–71. doi: 10.3390/d2010047.

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT (OECD) - OECD Guidline for the testing of chemicals. 2008) 1–33.

PROESTOU, Dina; FLIGHT, Patrick; CHAMPLIN, Denise; NACCI, Diane. - Targeted approach to identify genetic loci associated with evolved dioxin tolerance in Atlantic killifish (*Fundulus heteroclitus*). **BMC evolutionary biology**. ISSN 1471-2148. 14:1 (2014) 7. doi: 10.1186/1471-2148-14-7.

PUSCHENDORF, Robert; HODGSON, Lauren - Underestimated ranges and overlooked refuges from amphibian chytridiomycosis. **Diversity and Conservation** (2013) 1313–1321. doi: 10.1111/ddi.12091.

REED, D. H.; FRANKHAM, R. - Correlation between fitness and genetic diversity. **Conservation Biology**. 17:1 (2003) 230–237.

RIBEIRO, Rui; LOPES, Isabel - Contaminant driven genetic erosion and associated hypotheses on alleles loss, reduced population growth rate and increased susceptibility to future stressors: an essay. **Ecotoxicology** (London, England). ISSN 1573-3017. 22:5 (2013) 889–99. doi: 10.1007/s10646-013-1070-0.

ROARK, Shaun; BROWN, Karen - Effects of Metal Contamination From Mine Tailings on Allozyme Distributions of Populations of Great Plains Fishes. **Environmental Toxicology and Chemistry**. ISSN 07307268. 15:6 (1996) 921–927. doi: 10.1897/1551-5028(1996)015<0921:EOMCFM>2.3.CO;2.

ROWE, CL; FREDA, J. - Effects of acidification on amphibians at multiple levels of biological organization. Ecotoxicology of amphibians and reptiles. SETAC (2000).

ROWE, G.; BEEBEE, TJC - Population on the verge of a mutational meltdown? Fitness costs of genetic load for an amphibian in the wild. **Evolution**. 57:1 (2003) 177–181.

SAKUMA, M. - Probit analysis of preference data. **Applied Entomology and Zoology**. 33:339–347 (1998).

SANTORO, A. - Bioaccumulation of Heavy Metals by Aquatic Macroinvertebrates Along the Basento River in the South of Italy. Water, Air, and Soil Pollution. ISSN 0049-6979. 201:1–4 (2008) 19–31. doi: 10.1007/s11270-008-9923-5.

SCHAT, H.; BOOKUM, W. M. TEN - Genetic control of copper tolerance in *Silene vulgaris*. **Heredity**. 68 (1992) 219–229.

SCHMELLER, DS; SCHREGEL, Julia; VEITH, Michael - The importance of heterozygosity in a frog's life. **Naturwissenschaften** (2007) 360–366. doi: 10.1007/s00114-006-0205-z.

SILLERO, N.; RIBEIRO, R. - Reproduction of *Pelophylax perezi* in brackish water in Porto (Portugal). **Herpetology Notes**. 3 (2010) 337–340.

SOBRAL, Olímpia; MARIN-MORALES, Maria Aparecida; RIBEIRO, Rui - Could contaminant induced mutations lead to a genetic diversity overestimation? **Ecotoxicology**. ISSN 09639292. 22:5 (2013) 838–846. doi: 10.1007/s10646-013-1079-4.

STUART, SN; CHANSON, JS; COX, NA - Status and trends of amphibian declines and extinctions worldwide. **Science**. 306 (2004) 1783–1786.

TABASHNIK, B. - Determining the Mode of Inheritance of Pesticide Resistance with Backcross Experiments. **Journal**

of economic enthomology. 1991) 703–712. doi: http://dx.doi.org/10.1093/jee/84.3.703.

VAN STRAALEN, Nico M.; TIMMERMANS, Martijn J. T. N. - Genetic Variation in Toxicant-Stressed Populations: An Evaluation of the «Genetic Erosion» Hypothesis. **Human and Ecological Risk Assessment: An International Journal.** . ISSN 1080-7039. 8:5 (2002) 983–1002. doi: 10.1080/1080-700291905783.

VENÂNCIO, Cátia; RIBEIRO, Rui; SOARES, Amadeu; LOPES, Isabel - Multiple Stressor Differential Tolerances: Possible Implications at the Population Level. **Plos One**. ISSN 1932-6203. 11:3 (2016) 2–10. doi: 10.1371/journal.pone.0151847.

WAKE, DB; VREDENBURG, VT - Are we in the midst of the sixth mass extinction? A view from the world of amphibians. **Proceedings of the National Academy of Sciences of the United States of America**. 105 (2008) 11466–11473.

WALLACE, M. E.; SWINEY, F. J. MAC - A major gene controlling warfarin resistance in the house mouse. **Journal of hygene**. 76 (1976) 173–181.

WIND, E. - Effects of Habitat Fragmentation on Amphibians: What Do We Know and Where Do We Go From Here? Proc. Biology and Management of Species and Habitats at Risk (1999) 885–894.

WIRGIN, I.; ROY, N; HAHN, M. - Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson river. **Science**. 331 (2011) 1322–1325.

WOOD, R. J. - Insecticide resistance: genes and mechanisms. In The Genetic Consequences of Man Made Change. Academic p ed. London: [s.n.]. (1981) p. 53-96.

YOUNG, Leah Bendell; HARVEY, Harold H. - Metal concentrations in crayfish tissues in relation to lake pH and metal concentrations in water and sediments. **Canadian Journal of Zoology**. ISSN 0008-4301. 69:4 (1991) 1076–1082. doi: 10.1139/z91-152.

CHAPTER 5.

Does historical exposure to metal contamination provide tadpoles a higher lethal tolerance to these chemicals?

Does historical exposure to metal contamination provide tadpoles a higher lethal tolerance to these chemicals?

Abstract

The major goal of this work was to assess if natural populations of amphibians exposed to metal contamination (mining effluents) may have acquired an increased tolerance to mercury or lead. To attain this major goal, two specific objectives were delineated: 1) determine if historical exposure to metal contamination caused an increase in the levels of oxidative stress, metallothioneins and metal burden in tadpoles inhabiting these sites comparatively to those inhabiting reference sites; 2) assess if tadpoles from historically metal impacted sites exhibited a higher lethal tolerance to metal contamination comparatively to those inhabiting reference sites. For this, tadpoles of *Pelophylax perezi* were collected at reference and metal contaminated (with mercury and lead) sites. Metal body burden, oxidative stress and levels of metallothioneins (MT) were assessed in these tadpoles. Additional tadpoles were collected at each sampling site and were exposed, in laboratory, to mercury or lead. The parameters mentioned above plus mortality were monitored at the end of the assay. Tadpoles, already living in contact with Hg and Pb mining effluents showed increased metal body burden, pointing out that Hg and Pb can be easily accumulated by *P. perezi* (in the field) tadpoles, which could be used as a model organism to assess metal exposure in aquatic habitats. Tadpoles coming from historically metal contaminated sites, did not show increased oxidative stress comparatively to reference tadpoles, but, the former (originated from Hg contaminated sites) exhibited higher MT levels than the latter ones. Results obtained from laboratory assays did not revealed differential tolerance to Hg or Pb among reference and metal impacted populations: no significant differences were observed in mortality or oxidative stress among populations. Interesting, the levels of MT in tadpoles of reference populations exposed in laboratory to control conditions were lower than: (i) their relatives collected in the field and (ii) the tadpoles collected at Pb historically impacted sites also exposed to control conditions in the laboratory. These results may suggest higher constitutive levels of MT in metal exposed populations, which may indicate an adaptation of these populations to metal contamination.

Keywords:

Pelophylax perezi, Mercury and Lead, Oxidative stress, Metal body burden.

Introduction

Amphibians are facing a global crisis (Kiesecker and Blaustein, 1995; Kiesecker et al., 2001; Stuart et *al.,* 2004), a lot of species being subject to the threats posed by, among others, habitat loss, pathogens or pollution (Fasola *et al*., 2015; Freda 1986; McKenzie and Peterson 2012; Rouse *et al*., 1999; Sparling *et al*. 2010). Sensitivity to pollutants is not the same across species (Bridges and Semlitsch 2000; Ferguson and Gilbert 1967; Semlitsch *et al*., 2000), tolerant ones may be able to cope with contamination-induced stress, and come to colonize mildly impacted sites (Johansson *et al*., 2001; Semlitsch *et al*., 2000; Sillero and Ribeiro 2010). Natural populations can be able to inhabit metal contaminated sites, resisting to low but permanent metals ions concentrations (Adlassnig *et al*., 2013; Hangartner *et al*., 2012; Marques *et al*., 2013). This can be achieved by inducible or constitutive metabolic mechanisms (Hua *et al.,* 2013; Laurila *et al.,* 2002; Pedrosa *et al.,* 2016; Scheiner 1993a, 1993b, Timmermans *et al.,* 2005), or can be due to directional selection; resulting in genetically determined tolerance mechanisms (Andren et *al.*, 1989; Gomez-Mestre and Tejedo, 2003; Rasanen et *al.*, 2003; Semlitsch et *al.*, 2000). Earlier toxicity studies showed that some populations, when allowed to acclimate to a certain contaminant, can better cope with a future pulse of that same contaminant (inducible tolerance in insects, Poupardin *et al.*, 2008, and in amphibians, Hua *et al*., 2013). In aquatic habitats, *Chironomus riparius* inhabiting historically metal polluted sites showed higher tolerance to cadmium (Pedrosa *et al*., 2016) and fish, like the rainbow trout, showed an increased tolerance to zinc after being chronically exposed to nearly lethal concentrations, with an increase in metallothionein levels (Anadu *et al*., 1989). Hua *et al.* (2013) sampled populations of wood frogs (*Lithobates sylvaticus*) at sites, located close to and far from agricultural fields, in which insecticide carbaryl was applied regularly. These authors found that exposing some eggs and hatchlings of wood frogs to sublethal concentrations of carbaryl would result in higher tolerance to a subsequent lethal concentration of the same insecticide. They found this mechanism to be true for populations afar from the agricultural fields; tolerance to carbaryl in those populations resulted inducible. On the other hand, the same authors discovered that tolerance to carbaryl was not inducible in wood frogs populations residing close to agricultural areas; however individuals belonging to those populations already showed an higher tolerance, thus, the authors concluded that in this case tolerance was a constitutive mechanism (Hua *et al.,* 2013). This process, called genetic assimilation, consists in a plastic response that would turn into a constitutively expressed physiological mechanism (Debat and David, 2001; Hua *et al*., 2013;).

Amphibians, exposed to metal contamination, can absorb and accumulate these metal ions in their tissues. Increasing metal body burden, denote accumulation in the body and can be used as indicator

of exposure to metal contamination (Burger and Snodgrass 2001; Jofré *et al.*, 2012; Marques *et al*., 2009, 2011; Loumbourdis *et al*., 2007; Sparling, 1996;). Furthermore, exposure to metal may cause oxidative stress and damage in organisms due to the production of reactive oxygen species (ROS) (Atli and Canli, 2007; Apel and Hirt, 2004; Casalino *et al*., 2006; Kamiński *et al*. 2007; Pandey *et al*., 2003; Stohs and Bagchi, 1995; Zocche *et al*., 2014). Follows that, physiological processes would be activated to deal with such ROS, by producing enzymes able to lower the oxidative stress (Atli and Canli, 2007; Stohs and Bagchi, 1995; Zocche *et al*., 2014). This condition can be analyzed by means of specific biochemical markers, such as: superoxide dismutase (SOD) (Bannister and Calabrese, 1987), glutathione peroxidase (GPx) (Flohé and Günzler, 1984) and malondialdehyde (MDA) (Draper, 1990). The SOD and GPx have a key role in antioxidant cell's mechanisms, they are produced when a high quantity of highly oxidized compounds is present, being signal of an occurring antioxidant response (Bannister and Calabrese, 1987; Flohé and Günzler, 1984). Malondialdehyde is a compound resulting from lipid peroxidation, thus a marker of the effect of oxidative stress (Draper, 1990). The expected outcome, due to individual's exposure to metal contamination would be high levels of SOD, GPx and MDA. Populations expressing a different physiologically constitutive mechanism of tolerance to metals, which could determine a higher tolerance to metals by decreasing the oxidative damage; may show low basal levels in the activity of oxidative stress enzymes (Borković-Mitić *et al*., 2016). The same authors discovered such a pattern for *Pelophylax ridibundus*, an anuran belonging to the same genus of the Perez's frog. Borkovic-Mitic *et al*. (2016) interpreted the observed decrease in oxidative stress markers levels, for *P. ridibundus* individuals sampled at metal contaminated sites, with the presence of a protective constitutive mechanism against high concentrations of metals (Borković-Mitić *et al*., 2016).

Concentration of MTs have been found to increase, due to exposure to metals, in amphibians (Dobrovoljc *et al*., 2012; Loumbourdis *et al*., 2007), as an inducible mechanism. Metallothionein are polypeptides, rich in cysteine, with the capability to bind metal ions (and have been reported to be involved in the processes of metal detoxification as well as UV rays, heat-shock, and oxidative damage processes in invertebrates and vertebrates (Agrawal *et al*., 2014; Anadu *et al*., 1989; Aly *et al*., 2014; Coyle *et al*., 2002; Ding *et al*., 2002; Dobrovoljc *et al*., 2012; Geffard *et al*., 2007; Loumbourdis *et al*., 2007; Timmermans *et al*., 2005; Ye *et al*., 2010). The expected outcome on MTs levels, due to metal exposure, would be that of higher MTs. Specifically, Dobrovoljc *et al.* (2012), suggested that metallothionein, binding Cd, Cu and Se ions, in *Necturus maculosus* and *Bufo bufo* indicate successful mechanism to cope both with short and long-term exposure to metals.

In this context, the present study aimed at assessing if populations of Perez's frogs, historically exposed to metal contamination coming from mining effluents in Castilla-la Mancha region, may

have acquired an increased tolerance to metal contamination. For this, two specific objectives were set: 1) determine if historical exposure to metal contamination caused an increase in the levels of oxidative stress, metallothioneins and metal burden in tadpoles inhabiting these sites comparatively to those inhabiting reference sites; 2) assess if tadpoles from historically metal impacted sites exhibit a higher lethal tolerance to metal contamination than those from reference sites.

Materials and methods

Study organism:

The Perez's frog *Pelophylax perezi* (López-Seoane, 1885) was selected as the model species to perform the present study. This frog is endemic and common in the Iberian Peninsula (Arnold and Ovenden, 2002; Bosch *et al*., 2013; Loureiro *et al*., 2008) and it lives in almost any freshwater habitat from rivers to irrigation ditches and temporary ponds (Arnold and Ovenden, 2002; Bosch *et al*., 2013). In general, it is considered a tolerant frog, since it can, among other features, tolerate temperatures from 3°C to 35°C (Almeida *et al*., 2001; Bosch *et al*., 2013;) and colonize eutrophic and contaminated habitats (Arnold and Ovenden, 2002; Marques *et al*., 2013; Sillero and Ribeiro, 2010); this makes it an ideal species for research in the field of evolutionary ecotoxicology.

Sampling sites:

The "Iberian pyrite belt" comprise a geological region located at the southern Iberian peninsula, being exploited for mining activities since the Roman Empire ages (Edmondson, 1989; Higueras *et al*., 2006), namely in south of Spain, in the locations of: Alcudia valley, Almadén and Horcajo (Province of Ciudad Real, Castilla-La Macha region, Spain). Since the year 2000 the Almadén mine is converted into a museum, but metals analysis in the soil still show high mercury levels, with an increasing gradient towards the mine. Almadén mine produced the largest amount of mercury ever processed in planet Earth (Higueras *et al*., 2006; Lindberg *et al*., 1978). The Alcudia Valley mines hosted the major lead producers in Spain during the second half of the 19th century (Rodríguez *et al*., 2009). The Horcajo and Alcudia valley lead mines have a long history of exploitation, to the end of the 20th century (Baranda, 1994; Reglero *et al*., 2008, 2009). Reglero *et al*. (2008) reported that the high lead contamination in the soils was able to be transferred into plants (grass like *Gramineae* or trees of the genus *Quercus*, among others) and animals (*Cervus elaphus*) (Reglero *et al*., 2008). In this context, metal-impacted sites, which receive either Hg or Pb rich effluents from Minas de Almadén, Minas de Alcudia and Minas de Horcajo, were selected for the present study. Perez's frog tadpoles were sampled at the Hg contaminated rivers Arroyo del Tamujar (HG1) and Rio Guadalmez "Pozoblanco" (HG2); and the Pb contaminatd rivers Arroyo de Valdefuentes (PB1) and Arroyo de la Ribera (PB2).

Pelophylax perezi tadpoles where also collected from three reference sites, in the same province (to better account for natural variations among populations), in Picon "Arroyo del Raso" (R1), Picon "abrevadero" (R2) and Rio Bullaque "Pantanos de los Jarales" (R3). All the sampling sites are located in the Castilla-La-Mancha region, in south-western Spain (Table.5.1; Fig.5.1).

Table.5.1: Localization and physico-chemical parameters measured at the selected sampling sites (Hg and Pb values refer to analysis of water samples conducted by Higueras *et al.,* 2006 and by Reglero *et al.,* 2008 in the same hydrographic basin). R1 – Picon Arroyo del Raso, R2 – Picon Abrevadero, R3 – Rio Bullaque, HG1 – Arroyo del Tamujar, HG2 – Rio Guadalmez, PB1 – Arroyo de Valdefuentes, PB2 – Arroyo de la Ribeira.

Location	**R1**	**R2**	**R3**	**HG1**	**HG2**	**PB1**	**PB2**
Conductivity ($\mu s/cm^3$)	811	586	472	673	195	202	153
Temperature (°C)	28.0	26.3	28.4	20.3	35.2	15.9	20.4
pH	8.10	8.77	9.75	7.85	10.01	8.54	8.4
O_2 (%)	82.6	156.0	172.3	39.8	195.0	65.9	95.9
Contamination	-	-	-	Hg 11.2 ng/l	Hg 11.2 ng/l	Pb 19.6 ng/ml	Pb 19.6 ng/ml
Coordinates	39°04'65.39"N 4°06'03.28"W	39°05'16.18"N 4°06'56.62"W	39°10'36.69"N 4°20'86.05"W	38°73'36.67"N 4°87'60.80"W	38°71'75.04"N 4°95'84.75"W	38°51'82.93"N 4°10'59.65"W	38°50'71.60"N 4°44'75.95"W

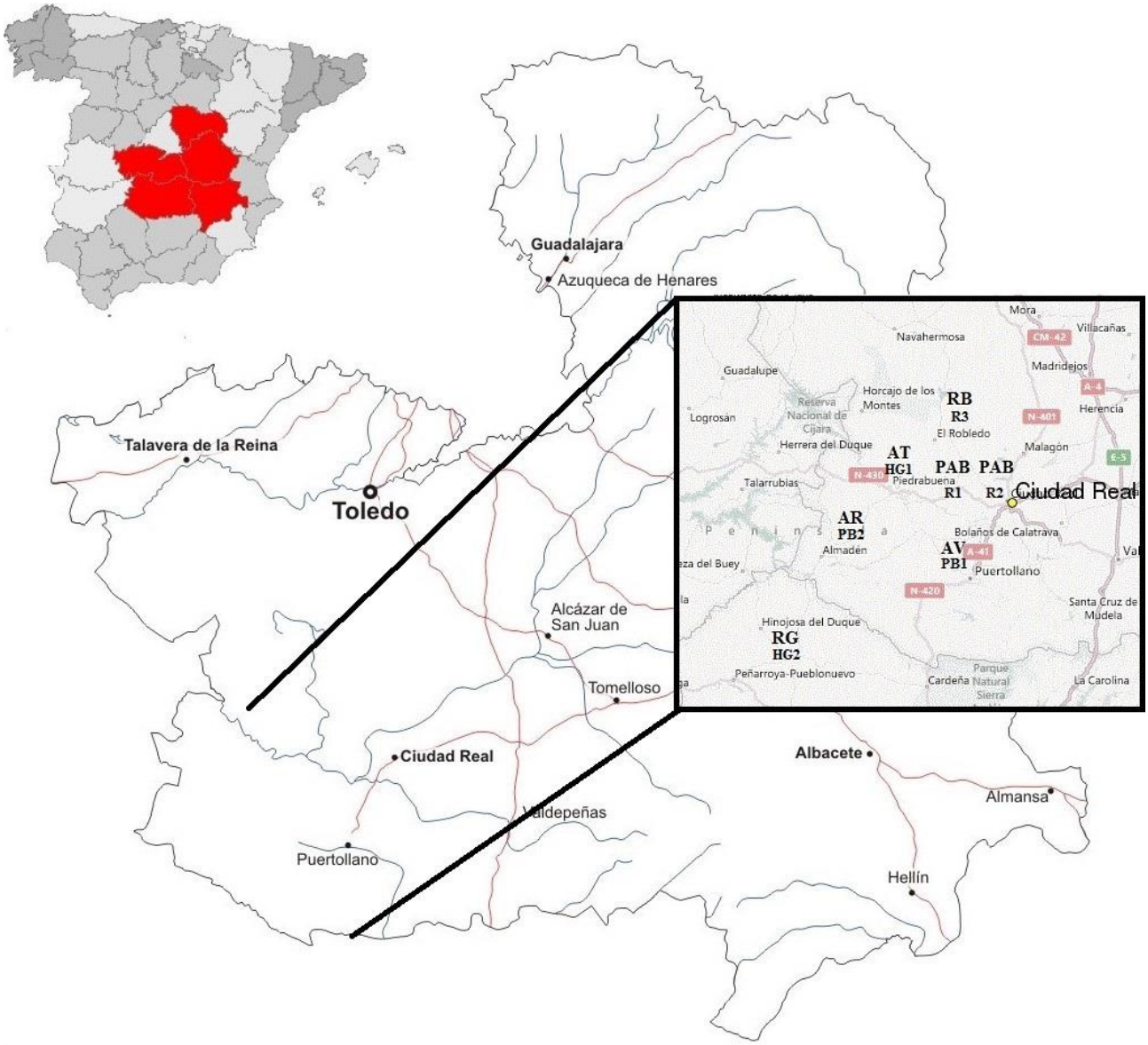


Fig.5.1: Map of the sampling sites; Picon "Arroyo del Raso" (PAR – R1), Picon "abrevadero" (PAB – R2), Rio Bullaque (RB – R3), Arroyo del Tamujar (AT – HG1), Rio Guadalmez (RG – HG2), Arroyo de Valdefuentes (AV – PB1) and Arroyo de la Ribera (AR – PB2).

Sampling and experimental design:

A total of 177 tadpoles were collected: 32 at R1, 3 at R2, 41 at R3, 28 at HG1, 4 at HG2, 30 at PB1 and 39 at PB2. From each sampling site (except for R1 and HG2), 20 tadpoles were introduced in plastic vessels, filled with their own pond water; and then transported to the laboratory in thermal containers to maintain an adequate temperature (following the rules for good practices in the transportation of research animals). The remaining tadpoles of each site were immediately deep frozen in liquid nitrogen (procedure done in the field) until arrival in laboratory where they were transferred to a -80ºC deep freezer until being used to determine biochemical markers, and metal body burden analysis.

Toxicity assay:

Upon arrival to the laboratory, the 20 tadpoles, for each site, were maintained in laboratory conditions for one week, at 23°C, in tanks filled with their respective pond water (which was also collected in the field), during this period they were fed *ad libitum* with dry fish food. After this period, tadpoles were used to perform lethal toxicity assays. Tadpoles from the reference sites (R1 and R3) were exposed to mercury (Hg) (1.5 mg/L) or lead (Pb) (10.5 mg/L), tadpoles from each mercury contaminated site were exposed to mercury (1.5 mg/L); tadpoles from each lead contaminated site were exposed to lead (1.5 mg/L); and their respective controls; were performed in Frog Embryo Teratogenesis Assay-Xenopus (FETAX) medium. No food was provided to tadpoles during the assays. Exposure concentrations were selected to be higher than LC_{50} values and values provoking sublethal effects reported in past studies for other amphibian species (Chen *et al.*, 2006; Freda, 1991; Horne and Dunson, 1995; Khangarot and Ray, 1987; Lu *et al*., 2006; Sparling *et al*., 2006; Taylor *et al*., 1990; Todd *et al*., 2011; Vatnick *et al*., 1999). The tadpoles' exposure was run for 24 hours at 23°C in a conditioned chamber, under a 14h/10h light/dark photoperiod. Two tadpoles were introduced in each plastic test vessel filled with 250 ml of test solution, and five replicates were performed per treatment (in total 10 tadpoles were exposed per treatment). During exposure mortality was recorded every 8 hours. An organism was considered dead if no movement was observed for 15 seconds after being gently stimulated with a pipette. The test was set to be a lethal acute exposure: (i) to mimic a short but powerful contamination pulse (as often occurs in winter when mining effluents discharge into rivers or ponds), and (ii) because, in general, literature reports a higher lethal tolerance in populations historically exposed to metal contamination (Barata et al. 2000) (and more specifically as reported in *Daphnia magna* exposed to copper, cadmium and zinc (Agra *et al*., 2011; Lopes *et al*., 2005); or in *Chironomus riparius* facing mercury or cadmium exposure (Pedrosa et *al*., 2016, 2017); in the laboratory.

Chemical analysis:

Tadpoles were analyzed to quantify Hg and Pb concentration in the body as well to quantify the levels of antioxidant enzymes (GPx, SOD), lipid peroxidation (quantified as concentration of malondialdehyde – MDA), and metallothioneins MTs. The whole body of the tadpoles was homogenized in one volume of Milli-Q water. For small specimens, several individuals from the same site and treatment were pooled together. Homogenates were separated in three aliquots.

The aliquot used for quantification of oxidative stress biomarkers (i.e. antioxidant enzymes and lipid peroxidation) was homogenized with nine volumes of phosphate buffer pH 7.4 with EDTA. Antioxidant enzymes were analyzed in homogenate supernatants following the procedures described

in Reglero *et al.* (2009). Briefly, GPx was quantified using RANSEL kits (Randox Laboratories, Crumlin, UK), based on the method by Paglia and Valentine (1967), which consists of quantifying GPx as catalyzer of the oxidation of glutathione by cumene hydropheroyde. SOD was quantified using RANSOD kits. The procedure consists of generating superoxide radicals from the oxidation of xanthine with xanthine oxidase, and further reaction of those radicals with 2-(4-iodophenyl)-3-(nitrophenol)-5-phenyltetrazolium chloride to generate a red-colored Formazan-based product. Both enzyme quantifications were conducted using spectrophotometrical techniques in an automated analyzer A25 (Biosystems, Barcelona, Spain). MDA was quantified following the protocol by Agarwal and Chase (2002). MDA was extracted from samples using n-butanol and quantified by high-purity liquid chromatography (HPLC) with a mixture of methanol and 50 mM potassium monobasic phosphate (pH 6.8) at a 40:60 ratio (v/v) as mobile phase.

The aliquot used for the quantification of metallothioneins was homogenized in three volumes of buffer containing 0.5 M sucrose and 20 mM tris-HCl pH 8.4, to which antiproteolytics (i.e. leupeptine and PMSF) and a reducing agent (2-mercaptoethanol) were added. Metallothioneins were analyzed spectrophotometrically using the protocol by Viarengo *et al*. (1997) with modifications as referred in Aly *et al*. (2014). The protocol consists of quantifying metallothionein contents through the analysis of cysteine amino acids using the spectrophotometrical Ellman's reaction (Ellman, 1959) after removing proteins of high molecular weight. Known concentrations of glutathione were used to build a calibration curve. The final metallothionein contents were estimated from glutathione equivalents adapting the procedures of UNEP/RAMOGE (Unep/Ramoge 1999) for metallothionein quantification in mollusks to amphibian metallothionein characteristics. Ellman's reactions quantifies the number of sulfhydryl residues of the cysteines forming the peptide sequence. The number of cysteines and the molecular weight of the metallothionein have to be taken into account to estimate metallothionein final concentration from glutathione equivalents. With this purpose, the metallothionein of *Pelophylax esculentus* (a closely related species to *P. perezi*) was used as references. This metallothionein contains a total of 20 cysteines and has a molecular weight of 7347.195 (~7350) DA (Trinchella *et al*., 2012). Therefore, from the concentration in the homogenate derived from the optical density measurement (OD412, quantified as µM), the final concentration of metallothioneins (MT) in the sample was calculated with the following equation: MT (as ng/g tissue) = [(OD412 / 20) * 4.5 * 5 * 7350] / tissue weight, where 20 is derived from the cysteine content, 4.5 from the final dilution factor of the sample used in Ellman's reaction, 5 from the initial dilution factor of the tissue in the homogenate (one volume of tissue, one volume of water and three volumes of homogenizing buffer), and 7350 from the molecular weight of frog metallothionein.

The aliquot used to quantify Hg and Pb contents in tadpoles was dried in an oven at 60ºC for 48 hours and the dry residues were digested in nitric acid and hydrogen peroxide as reported in Ortiz-Santaliestra *et al*. (2015). Lead was analyzed using graphite-furnace atomic absorption spectroscopy (GF-AAS; AAnalyst800 with autosampler AS800, Perkin-Elmer), and mercury was processed using a mercury/hydride system (MHS-15, Perkin-Elmer, Whaltham, MA, USA) coupled to the AAS. Blanks and reference materials were run in each set of analyses and used for calculation of limits of detection and test recovery. The recovery of the analyzed compounds ranged from 83% to 106%. Corrections, based on recovery data were not taken into account for quantification. Detection limits, calculated as 3SD of peak integrations obtained from blanks, were all below 0.01 ng/ml.

Data analysis:

Data analysis was carried out using STATISTICA 8.0. All the parameters (mortality, biochemical markers levels and metal body burden) were checked for normal distribution fitting (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test). When data were normally distributed the parametric one-way ANOVA test was performed (GPx) to assess significant differences between field populations and between individuals exposed in the laboratory compared with the controls. While non-normal distributed parameters (mortality, SOD, MDA, MTs and metal body burden) were analyzed through Kruskal-Wallis ANOVA by ranks tests (one way ANOVA of ranks), followed by the Dunn's multiple comparison nonparametric test to determine differences between field populations and between individuals exposed in the laboratory relatively to the controls. The Spearman correlation was conducted between biochemical marker levels and metal body burden.

Results

Field collected tadpoles:

- **Biochemical markers**

Glutathione peroxidase levels, for tadpoles sampled in the field, did not result statistically different between reference and historically contaminated sites (p_{GPx} = 0.1414) (Fig.5.2). The same was observed for SOD levels (p_{SOD} = 0.2775) (Fig.5.2). MDA values also showed the same pattern (p_{MDA} = 0.2789) except there was a tendency for lower values at metal contaminated sites samples (Fig.5.2). Metallothionein levels did not differ statistically among sites except for R2, which was comparatively lower than all other sites, and for historically mercury contaminated sites (HG1 and HG2), which showed values remarkably higher than all other sites (p_{MTs} = 0.00001) (Fig.5.2). GPx levels were positively correlated with SOD levels ($p<0.01$).

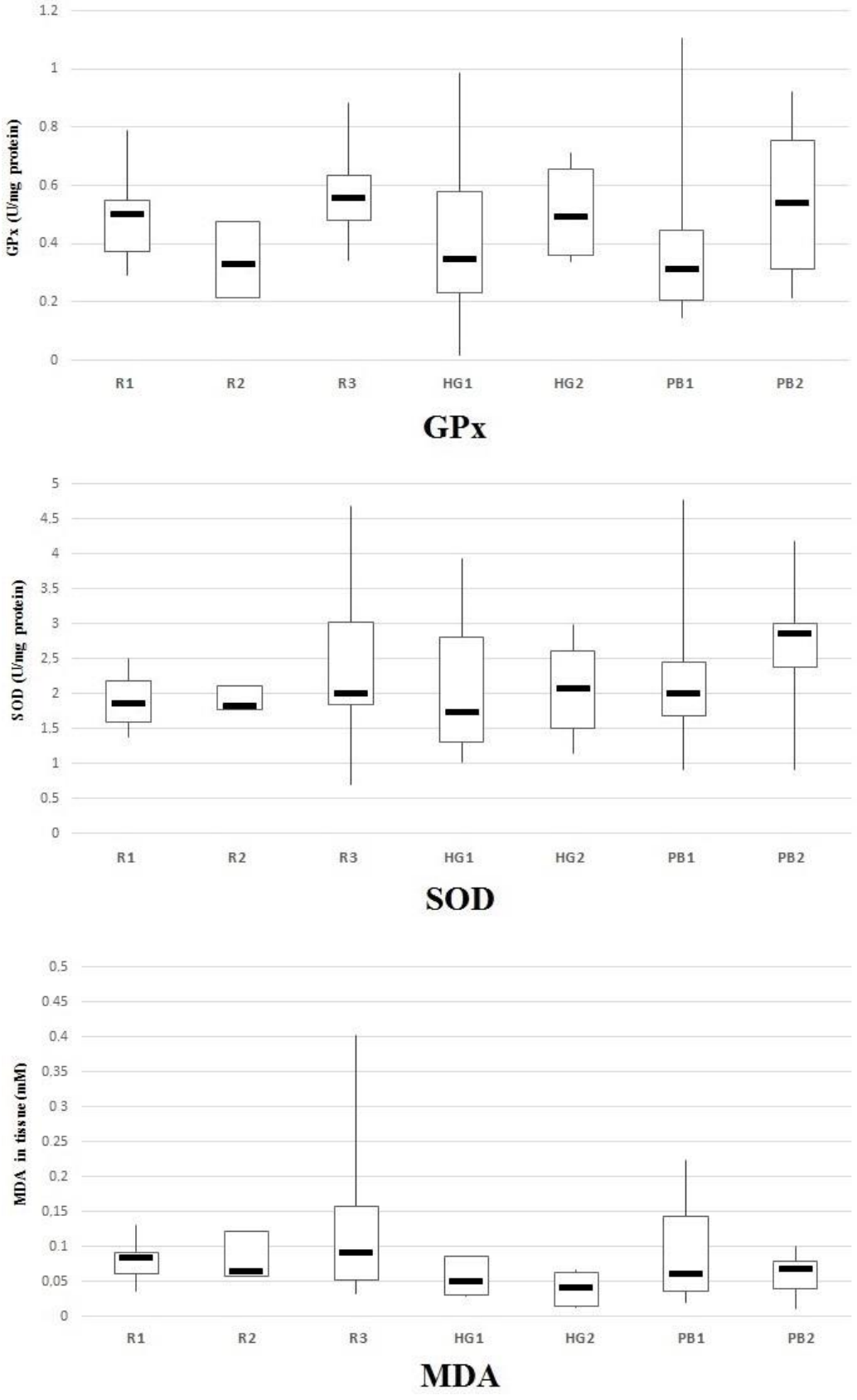


Fig.5.2: Levels of glutathione peroxidade (GPx), superoxide dismutase (SOD) and malondialdehyde (MDA) measured in tadpoles of *Pelophylax perezi* collected in the field at reference (R1, R2 and R3), Hg-contaminated (HG1 and HG2) and

Pb-contaminated (PB1 and PB2) sites. Thick lines indicate the median values, the box express the 25^{th} to 75^{th} inter-quartiles rage while thin vertical the line indicate minimum and maximum of the range.

- **Metal body burden**

The metal body burden was significantly different between reference and metal contaminated sites. Hg levels were higher in samples coming from Hg polluted sites (HG1 and HG2), compared to all other sites (p_{Hg} = 0.00001). Lead levels were markedly higher in samples coming from Pb contaminated sites (PB1 and PB2), compared to all other sites (p_{Pb} = 0.00001) (Fig.5.3). No significant correlations were detected between the metal body burden and the analyzed biochemical markers, except for MTs levels, which correlated positively with Hg content in the field samples (Spearman R = 0.4924, $p<0.01$).

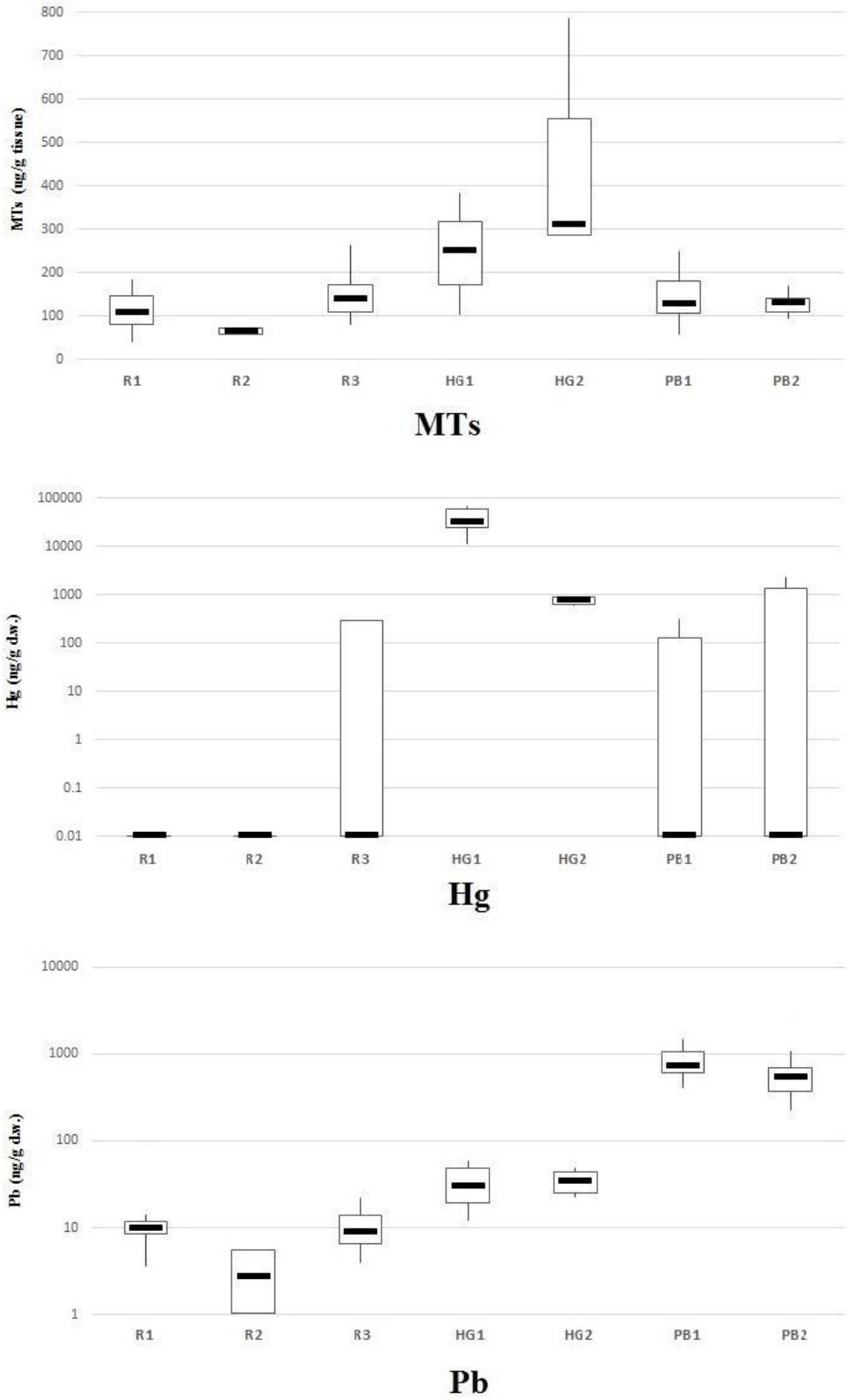


Fig.5.3: Levels of metallothionein (MTs) and metal body burden (Hg and Pb) measured in tadpoles of *Pelophylax perezi* collected in the field at reference (R1, R2 and R3), Hg-contaminated (HG1 and HG2) and Pb-contaminated (PB1 and PB2) sites. Thick lines indicate the median values, the box express the 25th to 75th inter-quartiles rage while thin vertical the line indicate minimum and maximum of the range.

Tadpoles from laboratory assays:

- **Mortality**

Time to death of tadpoles exposed to mercury, from HG1 (Hg impacted site) was not statistically different from those sampled at the reference sites (R1 and R3); however, a tendency in more delayed mortality was observed for historically exposed tadpoles (p = 0,09697; Fig.5.4). None of the tadpoles survived until the end of the exposure to mercury, on the other hand all control individuals remained alive. In the case of lead exposure, no lethal effect was observed, all individuals, both from reference (R1 and R3) and Pb contaminated sites (PB1 and PB2), remained alive through the duration of the test.

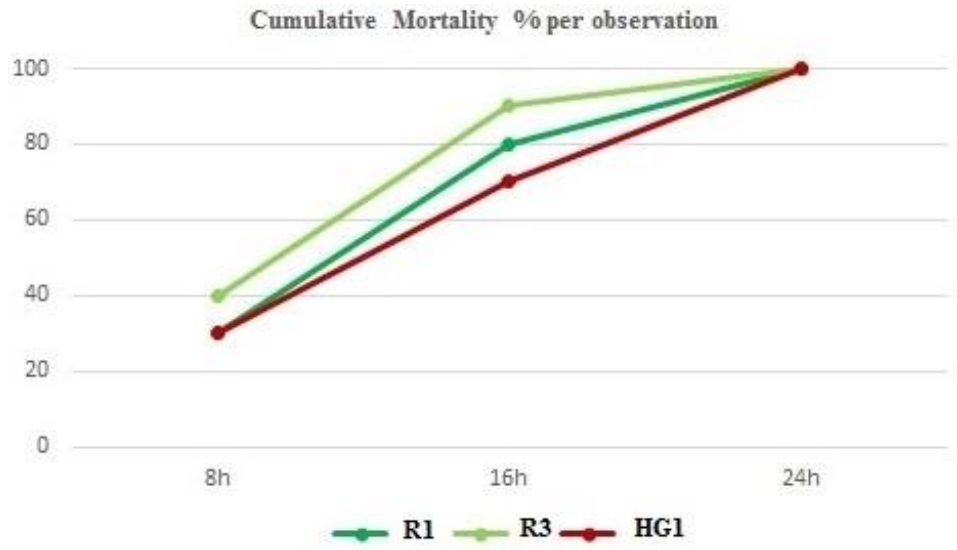


Fig.5.4: Cumulative mortality percentages of *Pelophylax perezi* tadpoles, per observation, exposed for 24h to lethal levels of mercury (green lines refer to reference sites, R1 and R3; while red line to a historically mercury polluted site, HG1).

- **Biochemical markers**

For control tadpoles, no significant differences were observed among sites for GPx, SOD and MDA levels. As well, no significant differences were detected between tadpoles exposed to Pb or to control, within each site (p_{GPx} = 0.7711, p_{SOD} = 0.7063, p_{MDA} = 0.1156). However, a tendency, though not significant, in SOD increase for R1 and R3 tadpoles exposed to Pb, and in SOD decrease for PB1 and PB2 Pb-exposed tadpoles was registered (Fig.5.5). Overall, for tadpoles exposed to Pb, no significant differences were observed among sites in the levels of GPx, SOD and MDA (p_{GPx} = 0.9110, p_{SOD} = 0.8779, p_{MDA} = 0.0630). Regarding the MTs, significantly higher levels of these proteins were measured, for PB1 and PB2 control tadpoles, comparatively to reference ones (p_{MTs} = 0.0046; Fig.6).

Compared to control tadpoles, the MTs levels showed a slight tendency to increase in Pb-exposed tadpoles from reference sites and a tendency to decrease in Pb-exposed tadpoles from historically metal contaminated site; this tendency was significant only for AV control tadpoles compared to their respective exposed tadpoles ($p_{MTs} = 0.0046$; Fig.5.6). GPx levels were positively correlated with SOD levels ($p<0.01$). Interestingly, when comparing MTs levels in reference tadpoles collected in the field (R1 and R3) to those exposed to control conditions in the laboratory, the former showed higher MTs levels than the latter ($p<0.01$) (Fig.5.3 and 5.6).

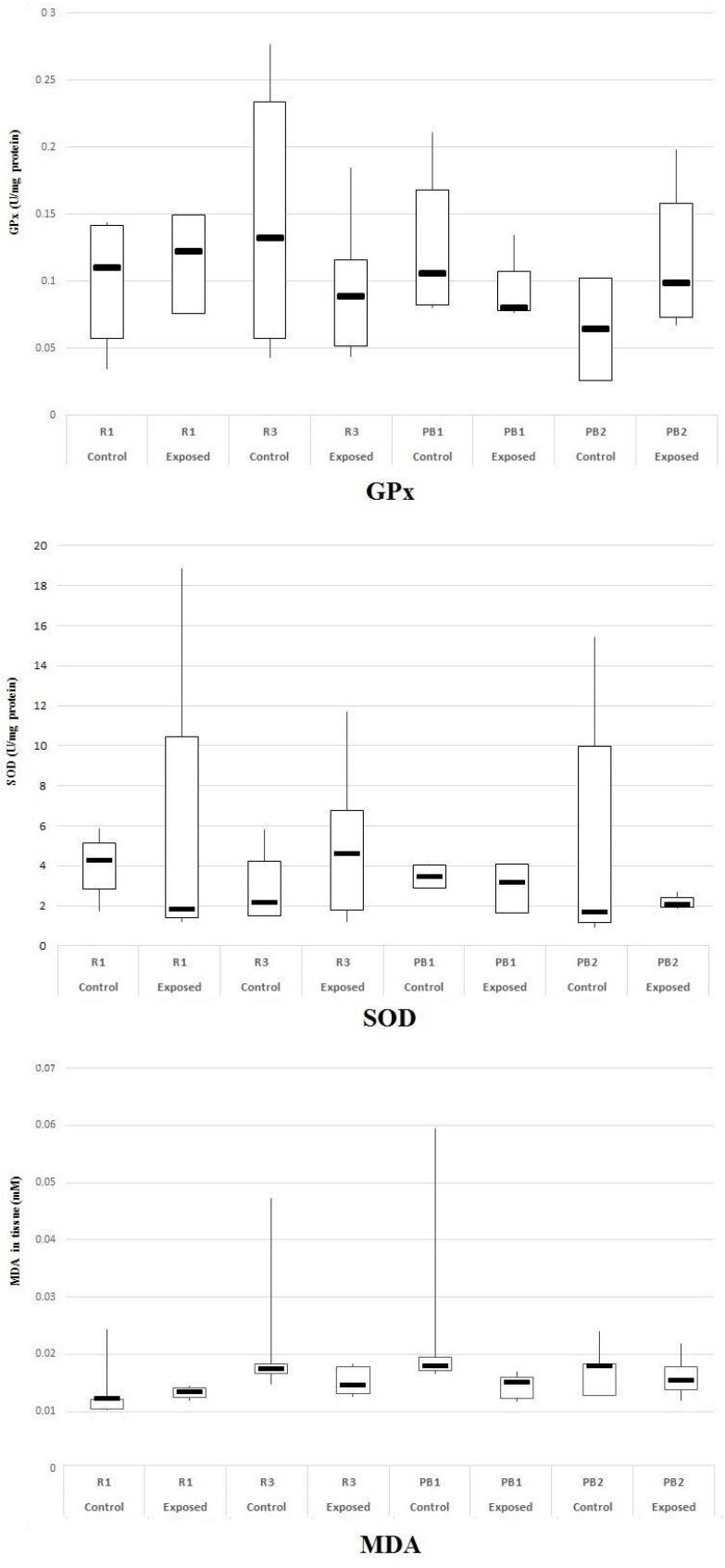


Fig.5.5: Levels of glutathione peroxidade (GPx), superoxide dismutase (SOD) and malondialdehyde (MDA) measured in tadpoles of *Pelophylax perezi* exposed to lead in the laboratory in comparison with the controls. Thick lines indicate the median values, the box express the 25th to 75th inter-quartiles rage while thin vertical the line indicate minimum and maximum of the range.

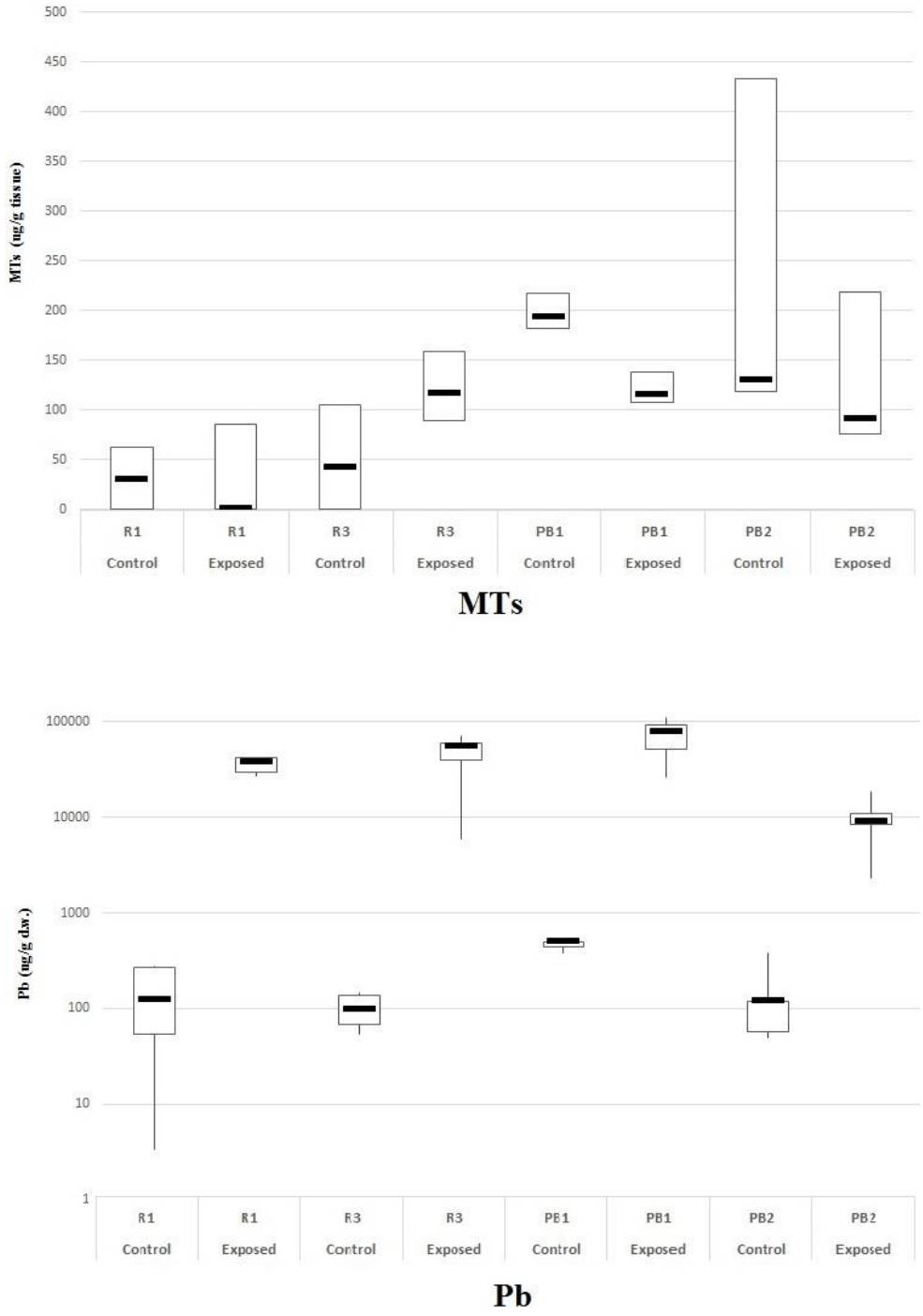


Fig.5.6: Levels of metallothionein (MTs) and metal body burden (Hg and Pb) measured in tadpoles of *Pelophylax perezi* exposed to lead in the laboratory in comparison with the controls. Thick lines indicate the median values, the box express the 25th to 75th inter-quartiles rage while thin vertical the line indicate minimum and maximum of the range.

- **Metal body burden**

The Pb body burden was significantly higher for tadpoles exposed to Pb, compared with their respective controls (p_{Pb} = 0.00001). Control tadpoles did show statistical differences between sites,

with PB1 samples containing higher Pb compared to controls from all other sites (p_{Pb} = 0.00001) (Fig.6). Furthermore, a comparison of Pb levels, from PB1 and PB2 tadpoles, sampled in the field and tadpoles exposed in laboratory control conditions revealed a significant decrease in the latter ($p_{Pbafterlab}$ = 0.0008). No significant correlations were detected between the metal body burden of tadpoles exposed to Pb and their respective analyzed biochemical markers levels ($p>0.01$).

Discussion

Field sampling:

Metal body burden analysis clearly revealed that Perez's frog populations at metal contaminated sites (HG1, HG2, PB1 and PB2) are indeed exposed to high levels of mercury and lead; as reported by previous studies at these mining areas (Higueras *et al*., 2006; Reglero *et al*., 2008). Compared to reference sites, the mercury and lead body burden levels were significantly higher at HG1 and HG2 (mercury contaminated) and PB1 and PB2 (lead contaminated sites), respectively. The present data clearly demonstrates that these metals are bioavailable to *P. perezi* tadpoles. This result is in line with previous studies indicating how metal body burden can be used as indicator of exposure to metal contamination, both for *P. perezi* and for other amphibians species (Burger and Snodgrass, 2001; Cooper and Fortin, 2010; Jofré *et al*., 2012; Marques *et al*., 2009, 2011; Sparling, 1996). Considering that the Perez's frog is: (i) able to colonize contaminated waters (Marques *et al*., 2013; Sillero and Ribeiro, 2010), (ii) is abundant and has no concerning conservation status (Arnold and Ovenden, 2002), (iii) tadpoles' collection in the field is relatively easy, and (iv) procedures to assess metal body burden are relatively simple; this species may be a suitable indicator to assess metal contamination in aquatic environments. However, the accumulation of Hg and Pb in tadpoles does not directly imply effects on their physiology as animals have mechanism that allow them, to some extent, to manage metal effects once incorporated, for example through induction of metallothioneins (Geffard *et al*., 2010; Unep/Ramoge, 1999). On the other hand, high mercury and lead body burdens in tadpoles may be of concern when they undergo metamorphosis; indeed, these metal ions can be remobilized and return bioavailable as the body of the tadpole goes through a process of extensive modification (Hofer *et al*., 2005).

Previous studies confirmed the increase of oxidative stress in *Xenopus laevis*, and *Rana nigromaculata* exposed to low levels of metals (Wang and Jia, 2009; Yologlu and Ozmen, 2015). In *P. perezi*, increasing response to metal contamination has been detected, specifically only for GPx levels and only in lung tissues (Marques *et al*., 2011). Nevertheless, in the present work, GPx, SOD and MDA did not show to increase in individuals living in metal contaminated sites. This situation

could be explained by different physiological mechanisms that Perez' frog tadpoles could use to cope with stress, induced by metal contamination, in the field. For example, tolerance to aluminum, in *Oncorhynchus mykiss* and *Salvelinus fontanilis*, is probably due to alteration of the chemical characteristics of the gill's surface anionic groups (McDonald and Wood, 1993; Reid *et al*., 1991). The absorption of copper and cadmium, in *Perca flavescens*, had been showed to be subject to different tissue specific binding sites; with Cu binding preferentially at the gills' surface while Cd at the intestines walls (Klinck *et al*., 2007). These mechanisms, which involve the availability of binding sites for ions on the surface of the gills is also supported by the biotic ligand model (Di Toro *et a*l., 2001). The lack of increase in oxidative stress, for tadpoles coming from historically metal exposed populations, has been already reported for *P. ridibundus* (Borković-Mitić *et al*., 2016); a close relative of *P. perezi*. Borkovic-Mitic *et al*. (2016) suggested that, historically metal exposed populations, would not show high levels of oxidative stress, because individuals belonging to such populations could express a constitutive mechanism of tolerance to metals, due to acclimation to the metal contaminated waters (Borković-Mitić *et al*., 2016). This situation may be plausible also in *P. perezi* populations sampled in the present study.

Statistically significant differences were registered for MTs level in tadpoles from the Hg impacted site of HG2, compared to reference sites tadpoles, and higher levels (but not statistically significant) for HG1 (also contaminated by Hg), compared with the reference sites and the Pb contaminated sites. Metallothionein levels have been showed, in literature, to increase in amphibians exposed to metal contamination (Burger and Snodgrass, 2001; Dobrovoljc *et al*., 2012; Marques *et al*., 2009, 2011). In the present work, this situation was supported for historically mercury-contaminated sites. Literature shows how populations, historically exposed to metal contamination, exhibited higher tolerance to acute metal exposure; for example in *Daphnia magna* exposed to copper, cadmium and zinc (Agra *et al*., 2011; Lopes *et al.*, 2005); or in *Chironomus riparius* facing mercury or cadmium contamination (Pedrosa *et al.*, 2016, 2017); in the laboratory. Therefore, the individual belonging to these populations would not experience particularly high oxidative stress; because other physiological mechanisms could lower the quantity of bioavailable metal ions. This explanation could be valid also for the above-discussed GPx, SOD or MDA levels; in the light of the higher MTs levels found to be associated to historically mercury (but not lead) exposed populations, in the present work. Additionally, MTs levels, in this study, correlated positively with Hg (but not Pb) body burden in the field samples. This is in line with Loumbourdis *et al*. (2007) whom found positive correlation between MTs and Cd body burden in *Rana ridibunda* individuals exposed to Cr and Cd in a laboratory experiment, with Johansen (2012), who discovered positive a correlation between Pb body burden and MTs levels in *Rana temporaria* tadpoles sampled in the field (Johansen, 2013) and Cooper and

Fortin (2010), who found correlation between MTs levels in the liver of *Rana catesbeiana* and respective Cu and Cd body burdens.

Laboratory assays:

The present work shows that acute mercury exposure rapidly causes mortality in Perez's frog tadpoles. The tadpoles, exposed to acute levels of mercury, reacted without detectable site-dependent differences. However, acute exposure to lead did not result lethal to *P. perezi* tadpoles exposed in the present study. Even if the tadpoles were exposed to a Pb concentration higher to those used in previous studies (Chen *et al*., 2006; Freda, 1991; Haywood *et al*., 2004; Sparling *et al*., 2006; Wang and Jia, 2009); Sparling *et al.* (2006) exposed *Rana sphenocephala* tadpoles to 1.3 mg/L (or more) and obtained lethal response after two days (Sparling *et al*., 2006). *Rana clamitans* did not show lethal effects when exposed for a week to 1 mg/L of Pb (Taylor *et al*., 1990) and *Rana nigromaculata* also showed no mortality (but increased oxidative stress) when exposed for one month to 1.6 mg/L of Pb (Wang and Jia, 2009). Haywood *et al.* (2004) found lethal effects on *Xenopus laevis* tadpoles only when exposed to 0.9 mg/L of Pb (Haywood *et al*., 2004). An explanation could be that *P. perezi* is in fact more tolerant to Pb than other species.

The biochemical markers, obtained for tadpoles in the toxicity assays, revealed that individuals of Perez's frog, exposed to Pb in the laboratory, proved to be quite tolerant to this metal (independently from the site of provenience). No oxidative stress responses in the form of increased levels of GPx and SOD or oxidative stress effects by increased MDA, in Pb-exposed tadpoles was observed. Comparatively, effects on glutathione and malondialdehyde were detected in *Rana nigromaculata* exposed to 1.6 mg/L (Wang and Jia, 2009).

Metallothionein levels, in the present study, were higher for tadpoles sampled at metal contaminated (Pb) sites and exposed to control conditions. Additionally, MTs levels were lower for exposed tadpoles from the same sites (PB1 and PB2), compared to their respective controls (but this difference was significant only for PB1 individuals). On the other hand, a not significant tendency in MTs increase was encountered for tadpoles sampled at R3 reference site, versus its respective controls. The MTs levels of reference tadpoles, measured in the field, versus the levels measured for tadpoles coming from the same populations (R1 and R3) after two weeks in the laboratory and subsequent exposure to control conditions, were markedly higher. While this pattern was not found for tadpoles coming from metal impacted populations, which showed high MTs levels (comparable to those obtained for field samples) after being maintained under laboratory conditions and subsequent exposure to control conditions. The level of MTs, in the field, could be influenced by environmental factors other than contamination, at reference sites. A possible explanation, for these finding, could

be that tadpoles inhabiting metal contaminated sites produce higher basal constitutive levels of MTs (compared to reference sites tadpoles) as a result of historical exposure to metals.

Lead body burden data, following the acute laboratory exposure, showed that Pb was significantly bioavalable for the exposed tadpoles, independently from their provenience, being in line with the results of previous studies, for various metals, including: Hg, in *Pelophylax ridibundus* (Borković-Mitić *et al*., 2016), Cr in *Rana ridibunda* (Loumbourdis *et al*., 2007), Cd in *Bufo arenarum* (Perez-Coll and Herkovits, 1996), U in *P. Perezi* (Marques *et al*., 2011) and Pb and Cd in six different anuran species (Jofré *et al*., 2012). This finding highlights how *P. perezi* can be a reliable model organism to assess metal uptake subsequent an acute exposure. Additionally, tadpoles collected at metal contaminated sites were able to lower their Pb body burden levels (practically by a half) after just one week in laboratory conditions; this result is in line with the decrease of cadmium encountered by Dobrovoljc *et al.* (2003) in tadpoles of *Rana dalmatina*, previously exposed to cadmium and later allowed to eliminate it, in clean laboratory conditions (50% reduction in one week) (Dobrovoljc *et al*., 2003). This could point to the presence of a similarly efficient lead excretion mechanism in the Perez's frog.

Conclusion:

Perez's frog tadpoles inhabiting historically metal contaminated sites did not show an increase in oxidative stress; possibly because of a constitutive higher baseline metallothionein production mechanism. However, tadpoles from historically contaminated sites did not proved to be more tolerant than those from reference sites, because no differences in lethal responses were found. Additionally, metal body burden proved to be a reliable indicator both for chronic (Hg and Pb) and acute (Pb) exposure. Indicating that mercury and lead can easily be absorbed by Perez's frog tadpoles. Such a fact rise the concern for this kind of contamination in habitats suitable for the life of amphibians' species. However, this outcome also point out that *P. perezi* can be used as a model organism to assess both acute and chronic exposure to mercury and lead, in the field.

Aknowledgments

This work was supported by funding FEDER through COMPETE - Programa Operacional Factores de Competitividade and by FCT Fundação para a Ciência e Tecnologia through project: (PTDC/BIA-BIC/3488/2012), within the CESAM's strategic programme (UID/AMB/50017/2013), as well with funds from the Castilla-La Mancha University and the doctoral fellowship of Emanuele Fasola (SFRH/BD/88955/2012). The authors would like to thank: Catia Venancio and Sara Costa for their support during fieldwork; Abel Ferreira for his help during lab work and Miguel Oliveira for his valuable comments.

References

ADLASSNIG, Wolfram *et al.* - Amphibians in metal-contaminated habitats. **Salamandra**. 49:3 (2013) 149–158.

AGARWAL, R.; CHASE, S. D. - Rapid, fluorimetric–liquid chromatographic determination of malondialdehyde in biological samples. **Journal of Chromatography**. B:775 (2002) 121–126.

AGRA, Ana Raquel; SOARES, Amadeu M. V. M.; BARATA, Carlos - Life-history consequences of adaptation to pollution. «*Daphnia longispina* clones historically exposed to copper». **Ecotoxicology**. ISSN 09639292. 20:3 (2011) 552–562. doi: 10.1007/s10646-011-0621-5.

AGRAWAL, S. *et al.* - Comparative oxidative stress, metallothionein induction and organ toxicity following chronic exposure to arsenic, lead and mercury in rats. **Cellular and Molecular Biology**. ISSN 1165158X. 60:2 (2014) 13–21. doi: 10.14715/cmb/2014.60.2.3.

ALMEIDA, N. *et al.* - Guia FAPAS dos anfíbios e répteis de Portugal. Porto, Portugal : FAPAS, 2001

ALPERS, C. N.; NORDSTROM, D. K.; SPITZLEY, J. - Extreme acid mine drainage from a pyritic massive sulfide deposit: The Iron Mountain end-member. In JAMBOR, J. L.; BLOWES, D. W.; RITCHIE, A. I. M. (Eds.) - Environmental Aspects of Mine Wastes. [S.l.] : Mineralogical Association of Canada Short Course Volume 31, 2003. p. 407–430.

ALY, W.; WILLIAMS, I. D.; HUDSON, M. D. - Limitations of metallothioneins in common cockles (*Cerastoderma edule*) and sponges (*Haliclona oculata*) as biomarkers of metal contamination in a semi-enclosed coastal area. **Science of the Total Environment**. ISSN 18791026. 473–474:2014) 391–397. doi: 10.1016/j.scitotenv.2013.11.136.

ANADU, D. I. *et al.* - Effect of zinc exposure on subsequent acute tolerance to heavy metals in rainbow trout. **Bulletin of Environmental Contamination and Toxicology**. 43:3 (1989) 329–336. doi: 10.1007/BF01701865.

ANDREN, C.; MARDEN, M.; NILSSON, G. - Tolerance to low pH in a population of moorfrogs, Rana arvalis, from an acid and a neutral environment: a possible case of rapid evolutionary response to acidification. **Oikos**. 56 (1989) 215–223.

APEL, K.; HIRT, H. - Reactive oxygen species: metabolism, oxidative stress, and signal transduction. **Annu. Rev. Plant Biol**. 55 (2004) 373–399.

ARNOLD, Nicolas; OVENDEN, Denys - Reptiles and Amphibians of Britain and Europe. [S.l.] : Collins; New edition, (2002).

ATLI, G.; CANLI, C. - Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus*. **Comp. Biochem. Physiol**. C:145 (2007) 282–287.

BANNISTER, J. V.; CALABRESE, L. - Assays for SOD. **Methods Biochem. Anal.** 32 (1987) 279–312.

BARANDA, Borja Sainz - The Horcajo mines. **Mineralogical Record**. 25:1 (1994) 1–21.

BARATA, Carlos *et al.* - Do Genotype Responses Always Converge From Lethal To Nonlethal Toxicant Exposure Levels? Hypothesis Tested Using Clones of *Daphnia Magna* Straus. **Environmental Toxicology and Chemistry**. ISSN 07307268. 19:9 (2000) 2314–2322. doi: 10.1002/etc.5620190922.

BLOWES, D. W. *et al.* - The geochemistry of acid mine drainage. In LOLLAR, B. S. (Ed.) - Environmental Geochemistry. [S.l.] : Treatise on Geochemistry, 2003. p. 149–204.

BORKOVIĆ-MITIĆ, Slavica S. *et al*. - Biomarkers of oxidative stress and metal accumulation in marsh frog (*Pelophylax ridibundus*). **Environmental Science and Pollution Research**. ISSN 16147499. 23:10 (2016) 9649–9659. doi: 10.1007/s11356-016-6194-3.

BOSCH, J. *et al*. - *Pelophylax perezi*. [On line], available at: www.iucnredlist.org.

BRIDGES, CM; SEMLITSCH, RD - Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. **Conservation Biology**. 14:5 (2000) 1490–1499.

BURGER, J.; SNODGRASS, J. - Metal levels in Southern Leopard Frogs from the Savannah river site: location and body compartment effects. **Environmental Research**. 86:2 (2001) 157–166. doi: 10.1006/enrs.2001.4245.

CASALINO, E. *et al*. - Acute cadmium intoxication induces alpha-class glutathione S-transferase protein synthesis and enzyme activity in rat liver. **Toxicology**. 217 (2006) 240–245.

CHEN, T. H.; GROSS, J. A.; KARASOV, W. H. - Sublethal effects of lead on northern leopard frog (*Rana pipiens*) tadpoles. **Environ. Toxicol. Chem.** 25 (2006) 1383–1389.

COOPER, Sophie; FORTIN, Claude - Metal and metallothionein content in bullfrogs: Study of a whole watershed impacted by agricultural activities. **Ecotoxicology and Environmental Safety**. ISSN 01476513. 73:3 (2010) 391–399. doi: 10.1016/j.ecoenv.2009.12.006.

COYLE, P. *et al.* - Cellular and Molecular Life Sciences Metallothionein : The multipurpose protein. 59 (2002) 627–647.

DEBAT, Vincent; DAVID, Patrice - Mapping phenotypes: canalization, plasticity and developmental stability. **Trends in Ecology & Evolution**. ISSN 01695347. 16:10 (2001) 555–561. doi: 10.1016/S0169-5347(01)02266-2.

DING, H. Q. *et al*. - Oxidative stress and metallothionein expression in the liver of rats with severe thermal injury. **Burns**. ISSN 03054179. 28:3 (2002) 215–221. doi: 10.1016/S0305-4179(02)00018-9.

DI TORO, D. M. *et al*. - Biotic ligand model of the acute toxicity of metals. 1. Technical basis. **Environmental toxicology and chemistry / SETAC**. ISSN 07307268. 20:10 (2001) 2383–2396. doi: 10.1897/1551-5028(2001)020<2383:blmota>2.0.co;2.

DOBROVOLJC, K.; JERAN, Z.; BULOG, B. - Uptake and elimination of cadmium in *Rana dalmatina* (Anura, Amphibia) tadpoles. **Bulletin of Environmental Contamination and Toxicology**. ISSN 00074861. 70:1 (2003) 78–84.

doi: 10.1007/s00128-002-0158-7.

DOBROVOLJC, Katarina *et al.* - Cd, Cu, Zn, Se, and metallothioneins in two amphibians, *Necturus maculosus* (Amphibia, Caudata) and *Bufo bufo* (Amphibia, Anura). **Biological trace element research**. ISSN 1559-0720. 150:1–3 (2012) 178–94. doi: 10.1007/s12011-012-9461-2.

DRAPER, H. H.; HADLEY H. - Malondialdehyde determination as index of lipid peroxidation. **Methods in Enzymology**. 186 (1990) 421–431.

EDMONDSON, J. C. - Mining in the later Roman Empire. **The Journal of Roman Studies**. 79 (1989) 84–102.

ELLMAN, G. L. - Tissue sulfhydryl groups. Arch. Biochem. **Biophys**. 82 (1959) 70–77.

FASOLA, E.; RIBEIRO, R.; LOPES, I. - Microevolution due to pollution in amphibians: A review on the genetic erosion hypothesis. **Environmental Pollution**. ISSN 18736424 02697491. 204:2015). doi: 10.1016/j.envpol.2015.04.027.

FERGUSON, D. E.; GILBERT, C. C. - Tolerances of three species of anuran amphibians to five chlorinated hydrocarbon insecticides. **J. Miss. Acad. Sci**. 13 (1967) 135–138.

FLOHÉ, L.; GÜNZLER, W. A. - Assays of glutathione peroxidase. **Methods Enzymol.** 105 (1984) 114–121.

FREDA, J. - The influence of acidic pond water on amphibians: A review. **Water Air Soil Pollution**. 20 (1986) 439–450.

FREDA, J. - The effects of aluminum and other metals on amphibians. **Environmental Pollution**. 71 (1991) 305–328.

GEFFARD, A *et al*. - Influence of biotic and abiotic factors on metallothionein level in Gammarus pulex. Comparative biochemistry and physiology. **Toxicology & pharmacology : CBP**. ISSN 1532-0456. 145:4 (2007) 632–40. doi: 10.1016/j.cbpc.2007.02.012.

GEFFARD, Alain *et al*. - Subcellular compartmentalization of cadmium, nickel, and lead in Gammarus fossarum: Comparison of methods. **Chemosphere**. ISSN 1879-1298. 78:7 (2010) 822–9. doi: 10.1016/j.chemosphere.2009.11.051.

GOMEZ-MESTRE, I.; TEJEDO, M. - Local adaptation of an anuran amphibian to osmotically stressful environments. **Evolution**. 57:8 (2003) 1889–1899.

HANGARTNER, Sandra; LAURILA, Anssi; RÄSÄNEN, Katja - Adaptive divergence in moor frog (*Rana arvalis*) populations along an acidification gradient: inferences from Q(st) -F(st) correlations. **Evolution; international journal of organic evolution**. ISSN 1558-5646. 66:3 (2012) 867–81. doi: 10.1111/j.1558-5646.2011.01472.x.

HAYWOOD, Lorren K. *et al*. - *Xenopus laevis* embryos and tadpoles as models for testing for pollution by zinc, copper, lead and cadmium. **African Zoology**. ISSN 1562-7020. 39:October (2004) 163–174. doi: 10.1080/15627020.2004.11657213.

HIGUERAS, P. *et al*. - The Almadén district (Spain): anatomy of one of the world's largest Hg-contaminated sites. **Science of the Total Environment**. ISSN 00489697. 356:1–3 (2006) 112–124. doi: 10.1016/j.scitotenv.2005.04.042.

HOFER, R.; LACKNER, R.; LORBEER, G. - Accumulation of toxicants in tadpoles of the common frog (*Rana temporaria*) in high mountains. **Archives of Environmental Contamination and Toxicology**. 49:2 (2005) 192–199.

HORNE, M. T.; DUNSON, W. A. - Effects of low pH , metals , and water hardness on larval amphibians. **Arch. Environ. Contam. Toxicol**. 29 (1995) 500–505.

HUA, Jessica; MOREHOUSE, Nathan I.; RELYEA, Rick - Pesticide tolerance in amphibians: Induced tolerance in

susceptible populations, constitutive tolerance in tolerant populations. **Evolutionary Applications**. ISSN 17524563. 6:7 (2013) 1028–1040. doi: 10.1111/eva.12083.

JOFRÉ, MB; ANTÓN, RI; CAVIEDES-VIDAL, E. - Lead and cadmium accumulation in anuran amphibians of a permanent water body in arid Midwestern Argentina. **Environmental Science and Pollution Research** (2012) 2889–2897. doi: 10.1007/s11356-012-0795-2.

JOHANSEN, SL - Element accumulation and levels of four biomarkers in common frog (*Rana temporaria*) tadpoles in two sedimentation ponds and a naturally occurring pond. [On line] avalable at: http://brage.bibsys.no/umb/bitstream/URN:NBN:no-bibsys_brage_42341/1/Master'sthesis Susanne Lund Johansen.pdf.

JOHANSSON, M.; RÄSÄNEN, K.; MERILÄ, J. - Comparison of nitrate tolerance between different populations of the common frog, *Rana temporaria***. Aquatic toxicology** (Amsterdam, Netherlands). 54:1–2 (2001) 1–14.

KAMIŃSKI, P.; KURHALYUK, N.; SZADY-GRAD, M. - Heavy metal-induced oxidative stress and changes in physiological process of free radicals in the blood of white stork (*Ciconia ciconia*) chicks in polluted areas. **Pollut. J. Environ. Stud**. 16 (2007) 555–562.

KHANGAROT, B. S.; RAY, P. K. - Sensitivity of toad tadpoles, *Bufo melanostictus* (Schneider), to heavy metals. **Bull. Environ. Contam. Toxicol**. ISSN 0007-4861. 38:3 (1987) 523–527. doi: 10.1007/BF01606623.

KIESECKER, Joseph M.; BLAUSTEIN, Andrew R. - Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. **Proceedings of the National Academy of Sciences of the United States of America**. 92 (1995) 11049–11052.

KIESECKER, Joseph M.; BLAUSTEIN, Andrew R.; BELDEN, L. K. - Complex causes of amphibian population declines. **Nature**. 410 (2001) 681–684.

KLINCK, J. S. et al. - Branchial cadmium and copper binding and intestinal cadmium uptake in wild yellow perch (*Perca flavescens*) from clean and metal-contaminated lakes. **Aquatic Toxicology**. ISSN 0166445X. 84:2 SPEC. ISS. (2007) 198–207. doi: 10.1016/j.aquatox.2007.03.024.

LAURILA, A.; KARTTUNEN, S.; MERILÄ, J. - Adaptive phenotypic plasticity and genetics of larval life histories in two *Rana temporaria* populations. **Evolution**. 56:3 (2002) 617–627.

LINDBERG, S. E. *et al.* - Atmospheric emission and plant uptake of mercury from agricultural soils near the Almadén mercury mine. **Journal of Environmental Quality**. 8:4 (1978) 572–578. doi: 10.2134/jeq1979.00472425000800040026x.

LOPES, Isabel; BAIRD, Donald J.; RIBEIRO, Rui - Genetically determined resistance to lethal levels of copper by *Daphnia longispina*: association with sublethal response and multiple/coresistance. **Environmental toxicology and chemistry / SETAC**. 24:6 (2005) 1414–1419.

LOUMBOURDIS, N. S. *et al*. - Heavy metal accumulation and metallothionein concentration in the frog *Rana ridibunda* after exposure to chromium or a mixture of chromium and cadmium. **Environmental pollution** (Barking, Essex : 1987). ISSN 0269-7491. 145:3 (2007) 787–792. doi: 10.1016/j.envpol.2006.05.011.

LOUREIRO, A. *et al*. - Atlas de Anfíbios e Répteis de Portugal. [S.l.] : INOVA, Portugal., 2008

LU, Xiang-yun *et al*. - Study on Joint Toxicity of Mercury ion and Copper ion to *Bufo gargarizans* Tadpoles. **Sichuan Journal of Zoology**. 2:2006).

MARQUES, Sérgio M. *et al*. - Antioxidant response and metal accumulation in tissues of Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine. **Ecotoxicology** (London, England). ISSN 1573-3017. 20:6 (2011) 1315–27. doi: 10.1007/s10646-011-0688-z.

MARQUES, Sérgio M. *et al*. - Differential gene expression in Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine. **Ecotoxicology and environmental safety**. ISSN 1090-2414. 87:2013) 115–9. doi: 10.1016/j.ecoenv.2012.10.010.

MARQUES, SM; ANTUNES, SC; PISSARRA, H. - Histopathological changes and erythrocytic nuclear abnormalities in Iberian green frogs (*Rana perezi* Seoane) from a uranium mine pond. **Aquatic Toxicology.** 91 (2009) 187–195. doi: 10.1016/j.aquatox.2008.04.010.

MCDONALD, D. G.; WOOD, C. M. - Branchial mechanisms of acclimation to metals in freshwater fish. In RANKIN, J.CLIFF; JENSEN, FRANK B. (Eds.) - Fish Ecophysiology Dordrecht : Springer Netherlands, (1993). doi.org/10.1007/978-94-011-2304-4_12>. ISBN 978-94-011-2304-4. p. 297–321.

MCKENZIE, VJ; PETERSON, AC - Pathogen pollution and the emergence of a deadly amphibian pathogen. **Molecular ecology** (2012) 5151–5154.

NORDSTROM, D. K. - Mine Waters : Acidic to Circumneutral. **Elements**. ISSN 1811-5209. 7:6 (2011) 393–398. doi: 10.2113/gselements.7.6.393.

NORDSTROM, D. K.; ALPERS, C. N. - Geochemistry of acid mine waters. In PLUMLEE, G. S.; LOGSDON, M. J. (Eds.) - The Environmental Geochemistry of Mineral Deposits. [S.l.] : Reviews in Economic Geology 6A, 1999. p. 133–160.

ORTIZ-SANTALIESTRA, Manuel E. *et al*. - Pollutant accumulation patterns in nestlings of an avian top predator: Biochemical and metabolic effects. **Science of the Total Environment**. ISSN 18791026. 538:2015) 692–702. doi: 10.1016/j.scitotenv.2015.08.053.

OVERNELL, J. - Occurrence of cadmium in crabs (*Cancer pagurus*) and the isolation and properties of cadmium metallothionein. **Environmental Health Perspectives**. ISSN 00916765. VOL. 65:10 (1986) 101–105. doi: 10.1289/ehp.8665101.

PAGLIA, D. E.; VALENTINE, W. N. - Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. **J. Lab. Cm. Med**. 70 (1967) 158–169.

PANDEY, S. *et al*. - Biomarkers of oxidative stress: A comparative study of river Yamuna fish Wallago attu (Bl. & Schn.). **Sci. Total. Environ**. 309:2003) 105–115.

PEDROSA, Joao *et al*. - The role of genetic diversity and past-history selection pressures in the susceptibility of *Chironomus riparius* populations to environmental stress. **Science of the Total Environment.** ISSN 18791026. 576:2017) 807–816. doi: 10.1016/j.scitotenv.2016.10.100.

PEDROSA, João *et al*. - Evolutionary consequences of historical metal contamination for natural populations of *Chironomus riparius* (Diptera: Chironomidae). **Ecotoxicology**. [s.d.]). doi: 10.1007/s10646-017-1784-5.

PEDROSA, João *et al*. - Investigating heritability of cadmium tolerance in *Chironomus riparius* natural populations: A physiological approach. **Chemosphere**. ISSN 00456535. 170:2016). doi: 10.1016/j.chemosphere.2016.12.008.

PEREZ-COLL, C. S.; HERKOVITS, J. - Stage-dependent uptake of cadmium by *Bufo arenarum* embryos. **Bull Environ**

Contam Toxicol. 56 (1996) 663–669.

POUPARDIN, R. *et al*. - Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito, *Aedes aegypti*: impact on larval tolerance to chemical insecticides. Insect **Biochemistry and Molecular Biology**. 38 (2008) 540–551.

RASANEN, K.; LAURILA, A.; MERILA, J. - Geographic variation in acid stress tolerance of the moor frog, *Rana arvalis* I. Local adaptation. **Evolution**. 57 (2003) 352–362.

REGLERO, M. M. *et al*. - Transfer of metals to plants and red deer in an old lead mining area in Spain. **Science of the Total Environment**. ISSN 00489697. 406:1–2 (2008) 287–297. doi: 10.1016/j.scitotenv.2008.06.001.

REGLERO, Manuel M. *et al.* - Heavy metal exposure in large game from a lead mining area: Effects on oxidative stress and fatty acid composition in liver. **Environmental Pollution**. ISSN 02697491. 157:4 (2009) 1388–1395. doi: 10.1016/j.envpol.2008.11.036.

REID, S. D.; MCDONALD, D. G.; RHEM, R. R. - Acclimation to Sublethal Aluminum: Modifications of Metal – Gill Surface Interactions of Juvenile Rainbow Trout (*Oncorhynchus mykiss*). **Canadian Journal of Fisheries and Aquatic Sciences**. 48:10 (1991) 1996–2005. doi: 10.1139/f91-238.

RODRÍGUEZ, L. *et al.* - Heavy metal distribution and chemical speciation in tailings and soils around a Pb-Zn mine in Spain. **Journal of Environmental Management**. ISSN 03014797. 90:2 (2009) 1106–1116. doi: 10.1016/j.jenvman.2008.04.007.

ROUSE, J. D.; BISHOP, C. A.; SRUGER, J. - Nitrogen pollution: an assessment of its threat to amphibian survival. **Environ. Health Perspect**. 107 (1999) 799–803.

SCHEINER, S. M. - Genetics and evolution of phenotypic plasticity. **Annual Review of Ecology and Systematics**. 24 (1993) 35–68.

SCHEINER, S. M. - Plasticity as a selectable trait: reply to Via. **American Naturalist**. 142 (1993) 372–374.

SEMLITSCH, R. D.; BRIDGES, C. M.; WELCH, A. M. - Genetic variation and a fitness tradeoff in the tolerance of gray treefrog (*Hyla versicolor*) tadpoles to the insecticide carbaryl. **Oecologia**. ISSN 0029-8549. 125:2 (2000) 179–185. doi: 10.1007/s004420000443.

SILLERO, N.; RIBEIRO, R. - Reproduction of *Pelophylax perezi* in brackish water in Porto (Portugal). **Herpetology Notes**. 3 (2010) 337–340.

SPARLING, D. W.; T.P., Lowe. - Metal concentrations of tadpoles in experimental ponds. **Evironmental pollution**. 91:2 (1996) 149–159. doi: 10.1016/0269-7491(95)00057-7.

SPARLING, Donald W.; KREST, Sherry; ORTIZ-SANTALIESTRA, Manuel - Effects of lead-contaminated sediment on *Rana sphenocephala* tadpoles. **Archives of Environmental Contamination and Toxicology.** ISSN 00904341. 51:3 (2006) 458–466. doi: 10.1007/s00244-005-0243-0.

SPARLING, DW *et al*. - Ecotoxicology of amphibians and reptiles (2010).

STOHS, S. J.; BAGCHI, D. - Oxidative mechanisms in the toxicity of metal ions. **Free Radic. Biol. Med.** 18 (1995) 321–336.

STUART, SN; CHANSON, JS; COX, NA - Status and trends of amphibian declines and extinctions worldwide. **Science**.

306 (2004) 1783–1786.

TAYLOR, D. H.; STEELE, C. W.; STRICKLER-SHAW, S. - Responses of green frog (*Rana Clamitans*) tadpoles to lead-polluted water. **Environmental Toxicology and Chemistry**. 9:1 (1990) 87–93. doi: 10.1002/etc.5620090111.

TIMMERMANS, Martijn J. T. N. *et al.* - Metallothionein mRNA expression and cadmium tolerance in metal-stressed and reference populations of the springtail *Orchesella cincta*. **Ecotoxicology**. ISSN 09639292. 14:7 (2005) 727–739. doi: 10.1007/s10646-005-0020-x.

TODD, Brian D. *et al.* - Aquatic and terrestrial stressors in amphibians: a test of the double jeopardy hypothesis based on maternally and trophically derived contaminants. **Environmental toxicology and chemistry / SETAC.** ISSN 1552-8618. 30:10 (2011) 2277–84. doi: 10.1002/etc.617.

TRINCHELLA, Francesca; ESPOSITO, Maria Grazia; SCUDIERO, Rosaria - Metallothionein primary structure in amphibians: Insights from comparative evolutionary analysis in vertebrates. **Biologies**. ISSN 16310691. 335:7 (2012) 480–487. doi: 10.1016/j.crvi.2012.05.003.

UNEP/RAMOGE - Manual on the biomarkers recommended for the MED POL biomonitoring programme. Athens : [s.n.]. ISBN 928071788X.

VATNICK, I. *et al.* - The effects of exposure to mild acidic conditions on adult frogs (Rana pipiens and Rana clamitans): mortality rates and pH preferences. **Journal of Erpethology**. 33 (1999) 370–374.

VIARENGO, Aldo *et al.* - A simple spectrophotometric method for metallothionein evaluation in marine organisms: An application to Mediterranean and Antarctic molluscs. **Marine Environmental Research**. ISSN 01411136. 44:1 (1997) 69–84. doi: 10.1016/S0141-1136(96)00103-1.

WANG, Mei Zhen; JIA, Xiu Ying - Low levels of lead exposure induce oxidative damage and DNA damage in the testes of the frog *Rana nigromaculata*. **Ecotoxicology**. ISSN 09639292. 18:1 (2009) 94–99. doi: 10.1007/s10646-008-0262-5.

YE, Boping *et al.* - Metallothioneins are required for formation of cross- adaptation response to neurobehavioral toxicity from lead and mercury exposure in nematodes. **PLoS ONE**. ISSN 19326203. 5:11 (2010). doi: 10.1371/journal.pone.0014052.

YOLOGLU, E.; OZMEN, M. - Low concentrations of metal mixture exposures have adverse effects on selected biomarkers of *Xenopus laevis* tadpoles. **Aquatic Toxicology**. 168 (2015) 19–27. doi: 10.1016/j.aquatox.2015.09.006.

ZOCCHE, J. J. et al. - Heavy-metal content and oxidative damage in *Hypsiboas faber*: the impact of coal-mining pollutants on amphibians. **Archives of Environmental Contamination and Toxicology**. 66:1 (2014) 69–77.

CHAPTER 6.

Environment and gender influences the skin microbiome composition of the Perez's frog.

Environment and gender influences the skin microbiome composition of the Perez's frog

Abstract:

Several microorganisms have been shown to live in association with amphibians, some of which being known for playing important roles in the protection of this group of vertebrates against infectious agents or pathogens (e.g. *Batrachochytrium dendrobatidis*). Within the scenarios of global changes, it is important to understand how environmental perturbations may adversely affect this microbiota since it may have consequences in the fitness of its host. According, this work aimed at assessing the influence of metal contamination in the diversity of the skin microbiome of the Perez's frog *Pelophylax perezi*. Swabs from male and female frogs' skin were collected at populations inhabiting metal contaminated and non-contaminated sites. Total microbial genomic DNA was extracted and used for DGGE and 16S rRNA gene-based metagenomics. The results suggest the division of the microbial community based on two factors: chemical contamination and gender of the frogs. The isolated cultivable bacterial strains were a total of 166 strains, DNA of each one was extracted and the 16S rRNA genes were sequenced for identification. All of the isolates were tested for the ability to grow against 3 acid mine drainage (AMD) concentrations (25%, 50% and 75%) plus in the LB medium. A core group of bacteria was identified in *P. perezi* skin microbiome, comprising the genera *Acinetobacter, Bacillus, Lactococcus, Microbacterium, Pseudomonas,* and *Stenotrophomonas*. The analysis, based in culture isolates, divides the microbial community based on two factors: chemical contamination of the sampling site and the gender of the frog. A significant inhibitory effect was observed subject to75% concentration of AMD, on bacterial isolates. No difference in tolerance to AMD was detected between strains coming from metal contaminated or non-contaminated sites. Frogs' skin microbial community differed by the presence or absence of environmental contamination and according to the frogs' gender, even within the same population.

Keywords: skin microbiome, acid mine drainage, amphibians, *Pelophylax perezi*.

Introduction

Many pluricellular eukaryotes on this planet live associated by a diversified microbiome. These microbiomes consists mostly of commensalists, if not beneficial for their host. Those interactions are complex and many times involve different mechanism that affect host's life cycles (Dobson *et al.*, 2012; Raven and Johnson, 2014; Salunke *et al.*, 2010).

Amphibians are a group of vertebrates that harbor a rich and diverse microbial community on their skin. This microbiome has been shown to be beneficial for this class of vertebrates, as they can enhance resistance to pathogens (Pask, 2012; Harris *et al.*, 2009; Becker *et al.*, 2009; Woodhams *et al.*, 2014; McKenzie and Peterson, 2012; Woodhams *et al.*, 2016; Laugen *et al.*, 2002; Bresciano *et al.*, 2015; Rebollar *et al.*, 2016), which are one of the major threats to amphibians nowadays (Blaustein *et al.*, 1994b; Fernández-Benéitez *et al.*, 2011; Baláž *et al.*, 2013; Pounds *et al.*, 2006; Pearman and Garner, 2005). The effectiveness of bacterial anti-pathogenic functions has been proved by experimental augmentation of the microbiome living in amphibians' skin (Harris *et al.*, 2009). Previous studies reported how the infection provoked by *Batrachochytrium dendrobatidis* alter the composition of the frog's skin microbial community (Bresciano *et al.*, 2015) and how the severity of the infection is season-dependent (Longo and Zamudio, 2016); because this fungus has it optimum growth temperature at 17-19 ºC, it is more virulent during the cold season (Stevenson *et al.*, 2013). The chytrid fungus is not the only pathogen that amphibians are facing, also *Saprolegnia* sp. water moulds and *Ranavirus*, are causing mass amphibians die-offs worldwide (Pounds *et al.*, 2006; Blaustein *et al.*, 1994a; Fasola *et al.*, 2015). All these agents may affect amphibians populations acting as co-occurring factors and adding a further risk of local extinction for amphibians populations already exposed to environmental pollution. Under scenarios of water quality deterioration, not only the amphibians inhabiting such polluted environment, but also the microbial community they harbor may be adversely affected. Consequently, if the polluted environment causes the loss of bacterial species diversity (by eliminating or decreasing the frequency of the most sensitive species) them some protection functions provided by the microbiome to the amphibian may render compromised (Woodhams *et al.*, 2014; McKenzie and Peterson, 2012; Woodhams *et al.*, 2016). Following this context, it becomes crucial to understand the factors structuring the skin microbial communities.

In some works amphibians microbiome was found to be species specific among different pristine sites (McKenzie *et al.*, 2012; Belden *et al.*, 2015; Rebollar *et al.*, 2016). This microbial community was recently shown to have seasonal shifts and to be influenced by the health status of the sampled amphibian (Longo and Zamudio, 2016; Bresciano *et al.*, 2015). Furthermore, the microbiome sampled onto amphibians was demonstrated to be neatly different from the bacterial associations

residing into the same ecological niches (Rebollar *et al.*, 2016). However, only one work, which investigated the same frog species studied in the present work, showed that contaminated habitats affects the amphibians skin microbiome (Costa *et al.*, 2016). Therefore, there is a lack of knowledge on the factors driving the composition of amphibians' skin microbiome, especially regarding the possible alterations caused by environmental contamination. This work assessed the influence of metal contamination in the diversity of the skin microbiome of the Perez's frog, exploring for the first time sex-dependent differences to assess if males and females frogs harbor the same microbial diversity. Possible differences on frogs' skin microbiome could be crucial to evaluate the differential impact of pollution and diseases on male and females.

Materials and methods

Sampling sites:

Four populations of *P. perezi* were sampled between March and June 2013, at four sites in Portugal. More than one reference site was considered to account for natural variability in the skin microbiome of frogs, caused by other factors rather than metal contamination. Three sampling sites were considered as reference [Barragem de Reguengos 1 (BR1): 37°51'38 N/ 8°13'56 W; Barragem de Reguengos 2 (BR2): 37°51'38 N/ 8°14'11 W; Lagoa do Cao (LC): 37°55'20 N/ 8°06'44 W]. The metal contaminated spot was [Ribeira da Água Forte (AF): 37°57'31 N/ 8°14'02 W] presenting high conductivity and low pH (Fig.6.1). This is a stream located in the Aljustrel mining area in the Iberian Pyrite Belt that drains the area of the old mining dumps, which has for decades been receiving acid mine drainage (AMD). This site was considered metal contaminated according to the water quality Portuguese legislation (Decree-Law 236/98).

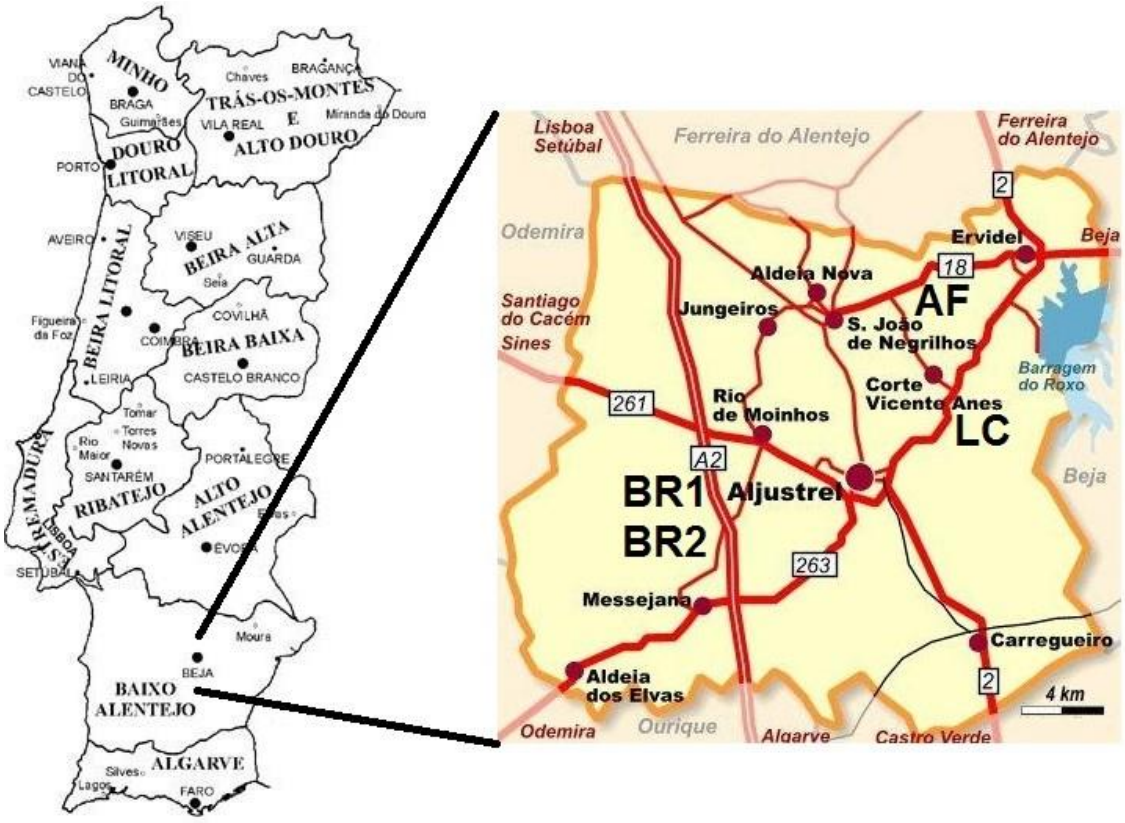


Fig.6.1: Map with the location of the sampling sites: Barragem de Reguengos 1 (BR1), Barragem de Reguengos 2 (BR2), Lagoa do Cao (LC) and Ribeira da Agua Forte (AF).

Acid mine drainage analysis:

For the analysis of total and dissolved trace metals, water was acidified with nitric acid SIGMA-ALDRICH® (65%), to a pH below 2, to prevent metal adsorption. The total concentrations of Al, As, Ca, Cd, Cr, Cu, K, Fe, Mg, Na, Ni, Pb and Zn were quantified in the AMD effluent through ICP-MS (µg/L). Resulting in 2,300 µg/L Al, 24 µg/L As, 95,000 µg/L Ca, 5.2 µg/L Cd, 0.7 µg/L Cr, 226 µg/L Cu, 18,000 µg/L Fe, 16,000 µg/L K, 43,000 µg/L Mg, 174,000 µg/L Na, 36 µg/L Ni, 10 µg/L Pb and 2,100 µg/L Zn. Conductivity (µS/cm), pH and dissolved oxygen (DO, mg/L) values were measured on site before water sampling using a LF 330/SET conductivity meter, a WTW330/SET-2 pH and OXI 330/SET dissolved oxygen meters. These parameters resulted in conductivity of 4,240 µS/L, pH 2.1 and DO 11,5 mg/L.

Collection of skin microbiome:

Considering all sites, 23 frogs were captured with an hand net, eight from AF, four from LC, nine from BR1 and two from BR2. They were handled with nitrile gloves, previously disinfected with ethanol (70 %). New nitrile gloves were used for each individual. Immediately before sampling, each frog was rinsed abundantly (dorsal and ventral side) with sterile distilled water to ensure the collection of skin-associated microbes rather than water-associated transient microbes (Lauer *et al.*, 2008). Skin

microbiome was collected using sterile swabs that were scrubbed along the animal, following the protocol described according to Brem *et al.* (2007). To standardize the procedure, each frog was swabbed five times along the length of ventral and dorsal region, head, lateral region, surface of thigh and paws. All the animals were maintained closed in a bucket for a limited amount of time to prevent double sampling and then released into their habitat after all the sampling. Each swab was placed in a sterile 1.5 mL tube with 50 µl of Nutrient Broth (NB) medium (Merck Millipore, Darmstadt, DE) and immediately stored in thermal cuvettes with ice until lab arrival, where they were transferred and stored in a deep-freezer (-80ºC) until further analysis. In total, were analyzed 16 frogs' skin swabs, eight frogs from the contaminated site (five males, three females) and eight frogs from the reference sites (four males, four females), which were chosen randomly. The random choice, alongside with balanced sexes ratio, were performed in order to assure sample representativeness of the *P. perezi* populations in the district of Aljustrel. Subsequently, 500 µl of NB medium were added to each tube containing swabs and 200 µl of this solution was inoculated into Petri dishes, with R2A agar medium (Difco Laboratories, Detroit, MI), to explore the cultivable fraction of the microbiome. One hundred µl of the each sample solution were put into a new sterile tube with 500µl of NB and 15 % glycerol, and stored at -80 °C, to be used for total DNA extraction and barcoding analysis.

DGGE analysis from *P. perezi* skin microbiome:

Total microbial genomic DNA was extracted from nutrient broth absorbed swabs (400 µl) and purified using the E.Z.N.A.® Soil DNA kit D5626-01 (Omega Bio-Tek). The 16S rRNA gene from total DNA obtained from frog swabs was amplified with the primers 341F (5′-CCT ACG GGA GGC AGC AG-3′) and 907R (5'-CCG TCA ATT CAT TTG AGT TT-3'). For DGGE profiling, a nested-PCR (amplicon from previous PCR was used as template) was performed using the primers 341F (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') 40-bp GC clamp and 534R (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer *et al.*, 1993). Both PCR were performed in 30µl reaction using Supreme NZYTaq DNA polymerase (1.5U, NZYTech); with 25 cycles: 1min at 95°C, 1min at 57°C, and 1min 30s at 72°C.

DGGE was conducted using a DCode™ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA). Amplicons from Nested-PCR were loaded into 8% polyacrylamide gels with a denaturing gradient ranging from 30% to 70% (100% denaturant is defined as 7M urea and 40% formamide). Gels were run at 70V for 17h at 60°C and stained with ethidium bromide. Then scanned using an image analyzer Quantity One 4.6.6 (Bio-Rad). The similarity between the microbiomes of the different frogs were determined based on the digitized DGGE profiles using the cluster analysis technique. The bands in each lane were detected manually. Similar bands for each lane were used to

produce a dendrogram by using the unweighted pairwise group method with arithmetic mean (UPGMA).

16S rRNA gene-based metagenomics of *P. perezi* skin microbiome:

Total DNA extracted above was used as template for 16S rRNA amplicon sequencing with MiSeq Reagent Kit v3 (Illumina, San Diego, USA). DNA of frogs' skin microbiome from contaminated and reference sites were pooled together, i.e., samples containing males from contaminated site (CM), females from contaminated site (CF), males from reference sites (RM) and females from reference sites (RF). The four samples were amplified using primers S-D-Bact-0008-a-S-16 and S-D-Bact-0343-a-A-15 (Klindworth *et al.*, 2013). Amplicons were verified for their correct size by gel electrophoresis. Bands with the expected size were excised and purified by PCR Purification Kit (Macherey-Nagel, Düren, Germany). Each PCR was performed in triplicates, which were pooled and analyzed by Bioanalyzer DNA 7500 Chip (Agilent, Santa Clara, USA). DNA quantification was performed by Picogreen (Thermo Fisher, Waltham, USA). Sequencing was performed using the paired end methods on a Illumina MiSeq (Illumina, Berlin, Germany). Later on, the sequences were trimmed and merged using Adapterremoval (Lindgreen, 2012) using default values. In the case of ambiguous bases at 5'/3', the termini were trimmed and scores equal or less than 15. Contaminating PhiX sequences were removed using DeconSeq v.0.4.3 (Schmieder and Edwards, 2011). Chimeric sequences and plastid sequences were removed by using QIIME v.1.9.1 (Caporaso *et al.*, 2010). OTU picking was done using Open-reference OTU picking against the 97% similarity clustering of Greengenes as of May 2013 (DeSantis *et al.*, 2006). Basic diversity analysis was likewise done using QIIME v.1.9.1 at a subsampling of 56,121 reads. Stacked barplots of the taxonomical distribution, filtering away all OTUs with less than 1% of reads per sample, was done using R v.3.2.4 (http://r-project.org). The PCA biplot was done on Hellinger transformed data filtering away all OTUs with less than 5% of reads per sample, also using R v.3.2.4. The following R packages were used: vegan (Oksanen *et al.*, 2016), gplots, gplots2, sciplot, plyr, MASS and gridExtra.

Isolation and quantification of bacterial density:

The sixteen frog skin microbiome samples were cultivated by spread plate technique on non-selective R2A agar medium (Difco Laboratories, Detroit, MI). From each microbiome community, 100µl were spread on R2A, in triplicate, and incubated at 22°C for 8 days. The number of colonies per plate was counted and the results were expressed in colony-forming units (CFU) per frog. Colonies from all different morphotypes, based on color, border, size, brightness and texture (Smibert RM & Krieg NR, 1994), were randomly isolated. From each sample of AF, LC, BR1 and BR2 growing on R2A were

isolated a total of 166 cultures. Those were sub-cultured for purification and preserved in NB-15 % glycerol (v/v) at -80 °C.

16S rRNA gene sequencing of bacterial isolates:

The DNA from bacterial pure cultures was obtained using the protocol from *Pitcher et al.* (1989). Amplification of the nearly full-length 16S rRNA gene sequence from each DNA was performed by PCR with the primers 27F (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1525R (5 ′-AGA AAG GAG GTG ATC CAG CC-3 ′) (Rainey *et al.*, 1996). The 30 µl PCR reaction mix contained: reaction buffer (1.5mM $MgCl_2$, 50mM KCl and 10mM Tris-HCl, pH 8.3), 100µM (each) deoxynucleoside triphosphates (Invitrogen), 0.2µM (each) primer, 1.5U Taq polymerase (Bioline) and 1.5 µL of purified DNA solution as template. The PCR was performed with 30 cycles: 1 min at 94 °C, 1 min at 55 °C and 1 min and 30 s at 72 °C. The PCR products with expected size were purified by using the NZYGelpure kit (NZYTech). DNA sequencing of bacterial isolates was performed using commercial sequencing facilities (Macrogen Inc., Europe) and quality of the sequences was verified by using Sequence Scanner v1.0 software (Applied Biosystems).

Phylogenetic analysis of bacterial isolates:

All obtained sequences were compared with those available in the EMBL/GenBank database using BLASTN network services (Altschul *et al.*, 1997) and with sequences in the Eztaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012) on the basis of 16S rRNA gene sequence data and phylogenetic analysis. The SINA alignment service was performed on all sequences (Pruesse *et al.*, 2012). Sequences were then included in 16S rRNA-based Living Tree Project (LTP) release 115 database (http://www.arb-silva.de/projects/living-tree/) by parsimony in ARB software version 5.5 (Ludwig *et al.*, 2004). Phylogenetic dendrograms of sequences from this study and closest reference sequences were constructed by the Randomized Axelerated Maximum Likelihood (RAxML) method with GTRGAMMA model (Stamatakis, 2006) included on ARB software (Ludwig *et al.*, 2004). Bootstrap analysis with 1,000 replicates was used to evaluate the robustness of the phylogeny. Species based on 16S rRNA gene sequence were defined at 97% similarity or higher when clustering with the closest type strain in the phylogenetic tree.

Effluent toxicity assay:

All of the 166 isolated strains were tested, for growth rate, against three AMD concentrations (25%, 50% and 75%) plus a control (distilled autoclaved water). The toxicity of pure AMD (100%) could not be tested for two reasons: i) the frogs never live at such an high metal and hydrogen ion

concentrations, making this an unrealistic scenario, ii) pure AMD concentration could not be achieved because of the inoculated amount of bacterial cells, which should have necessarily been in non-contaminated liquid medium.

For inoculation in the toxicity assay, a suspension of each strain was produced in LB medium. Tests were conducted into 5 ml glass vessels. Each vessel was inoculated with 600 µl of LB medium and with 400 µl of LB medium containing an amount of cells suspension sufficient to make its optical density (OD_{600nm}) up to 0.4. AMD dilutions with distilled autoclaved water were added in a total of 3 ml (to the 1 ml LB already in the vessel). The controls were performed adding 3 ml of autoclaved distilled water, AMD's 25%, 50% and 75% concentrations were obtained by dilution with the same autoclaved distilled water. Each strain was tested in duplicate. All the flasks were sealed, shacked at 100rpm and incubated at 22 °C for 48h. Optical densities were measured, at 600 nm, using a Jenway 6405 UV Spectrophotometer, Keison International Ltd.

Data analysis:

Diversity indices were computed for bacterial isolates and for barcoding results based on the number of strains belonging to each phylotype (genera) identified in this study. DGGE bands at the same position were considered members of the same OTU for diversity calculations. PAST 2.08 software (Hammer *et al.*, 2001) was used to calculate the Shannon–Weaver index (H′) and Simpson indices, and similarity percentage (SIMPER) and perform one-way ANOVA.

Based on the results of OTUs obtained by sequencing of the obtained amplicons, alpha diversity was calculated, including Chao1 (QIIME package), as well as Shannon and the Simpson indices (PAST 2.08 software). To estimate Beta diversity, Bray-Curtis dissimilarity matrix were used to examine the similarity of the membership and structure found in the various samples.

Redundancy analysis (RDA) was performed in order to reveal relationships between frog's gender, bacterial species and the presence of contamination by using the software package CANOCO (version 4.5.1). A Monte Carlo permutation tests was performed to evaluate the statistical significance of the effects of the explanatory variables on the species composition of the samples (van den Brink and Braak, 1999).

Venn diagrams were constructed using the bioinformatic tool at http://bioinformatics.psb.ugent.be/cgi19 bin/liste/Venn/calculate_venn.htpl. While the results of the toxicity tests were analyzed with Statistica for Windows 8.0 (StatSoft, Aurora, CO, USA), by mean of a two-way ANOVA; all parameters' distributions were previously checked for normality using a Kolmogorov-Smirnof test. Additionally, we investigated similarities among bacterial strains with Primer 6.1.6 (PRIMER-E Ltd, 2006) and the following analyses were applied: non-metrical

multidimensional scale (NMDS, resemblance matrix costructed with the Jaccard's method), and a two-way similarity percentage analysis (SIMPER, using Bray-Curtis similarity method).

Nucleotide sequence accession numbers:

The 16S rRNA gene sequences of the bacterial isolates reported in this study were deposited in the Genbank database, under the accession numbers according KY611613 - KY611778. The sequences obtained from the amplification of the 16S rRNA gene using the extracted community DNA as template have been deposited GenBank under the SRA Accession numbers SRR5238458 (RM), SRR5238459 (RF), SRR5238460 (CM) and SRR5238461 (CF).

Results

DGGE analysis from *P. perezi* skin microbiome:

Each DGGE lane shows the results from one frog' skin microbiome, showing the pattern of diversity of the bacterial 16S rRNA genes present. The visual analysis of the DGGE patterns from all frog skin samples had heterogeneity, revealing bands common to both frogs from the same and different sampling sites. The total number of DGGE band positions detected in the gel was 32 and the average number of DGGE bands in samples from the contaminated site was 8 bands and 13 bands in samples from the reference sites (Fig.6.2).

The dendrogram generated from frogs' skin microbiome based on the DGGE profiling clustered in 4 groups (Fig.6.2). One group included DGGE's lanes 2, 4, 6 and 7 clustered together; all are male frogs from the contaminated site. Second cluster included lanes 3 and 5; both females from the contaminated site. A third cluster included lanes 13, 14, 15 and 16; three males and one female, all from the reference sites (with the males grouping together and the female being the closest neighbor). The fourth evident cluster included lanes 9, 10, 11 and 12; two males and two females, all from the reference sites and subdivided by gender (Fig.6.2). Given this DGGE structure, in the following 16S rRNA gene sequence NGS analysis, samples were pooled together in four groups by contamination and by frog's gender.

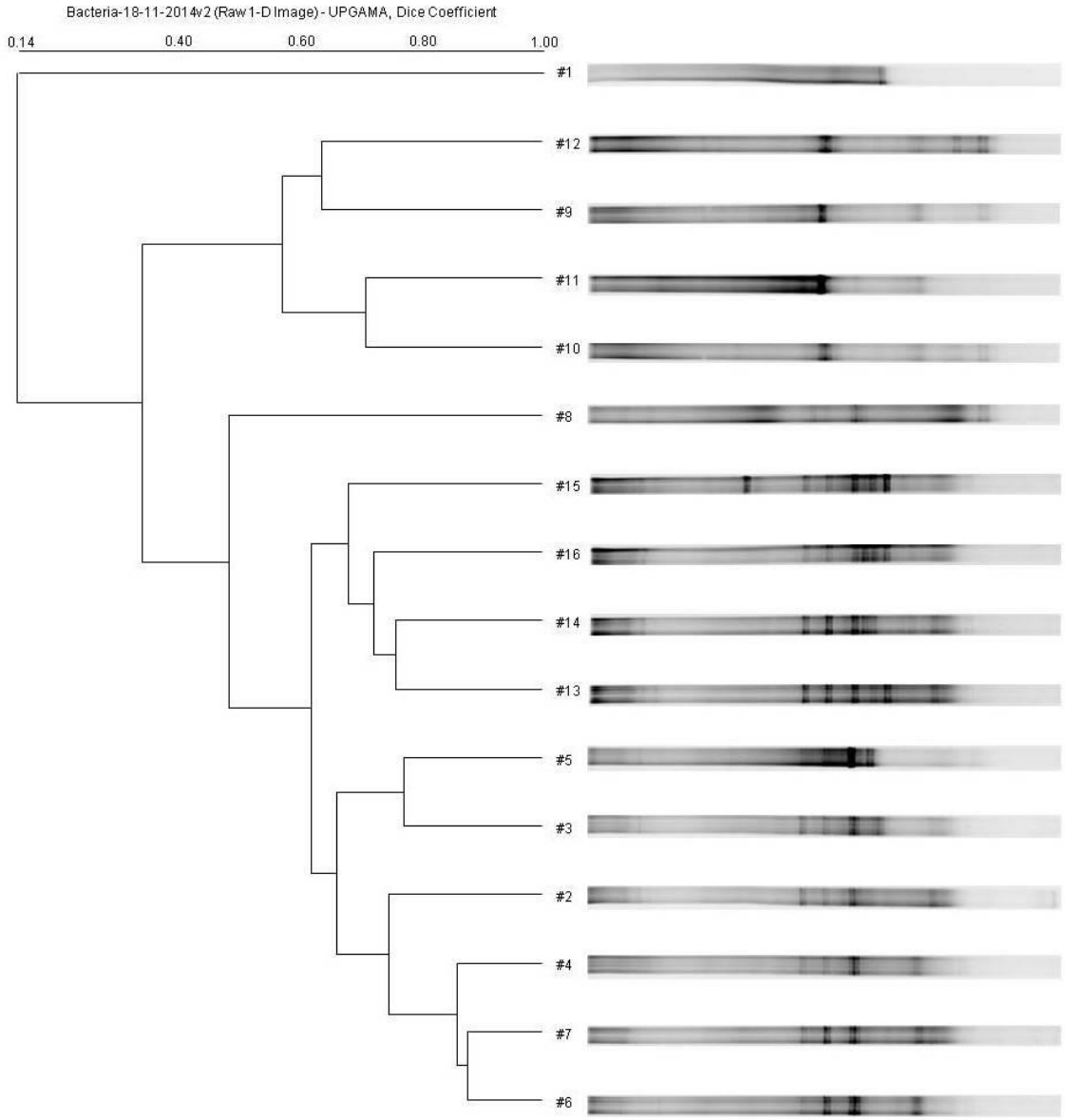


Fig.6.2: Similarity coefficient (UPGMA) between DGGE lanes deriving from *Pelophylax perezi* frog's skin bacterial samples. Lanes from 1 to 8 derive by samples from the contaminated site, lanes from 9 to 16 derive from reference sites (1,3,5,8,9,12 and 15 represent samples from females, and 2,4,6,7,10,11,13,14 and 16 from males).

Microbiome diversity by next generation sequencing of *P. perezi* skin community:

A total of 382,278 reads were generated by sequencing 16S rRNA gene based amplicons derived from total DNA from 4 samples covering male and female frogs both from metal contaminated and reference sites. The total number of reads ranged from 57,628 (RF) to 137,435 (CF). After filtering, reads ranged from 56,121 to 136,507 for RF and CF, respectively. Thus, for further analysis all reads from all sequenced samples were subsampled to 56,121 reads. After subsampling, the analysis of rarefaction curves showed for all samples sufficient sequencing depth to assess OTU diversity at a level of 97 % similarity (Fig.6.3). Representative sequences from each OTU were phylogenetically

classified using the Greengenes database. The number of detected OTUs ranged from 1,500 (CF) to 269,500 (CM).

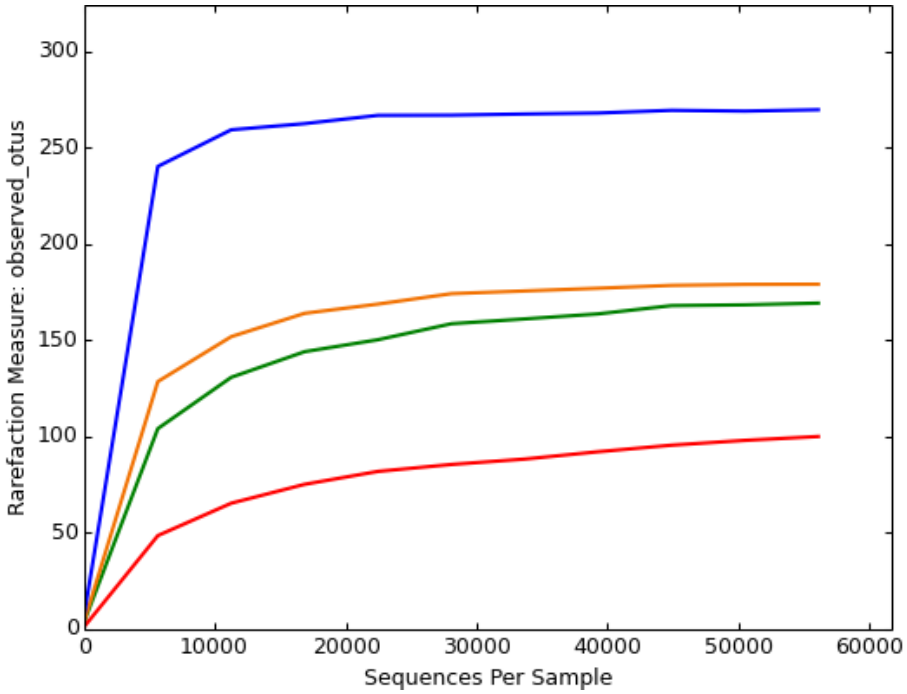


Fig.6.3: Rarefaction analysis curves of *Pelophylax perezi* skin bacterial communities. Sequences were clustered into OTUs using a sequence identity threshold of 97 %. (blue line = male frogs from contaminated site, red line = female frogs from contaminated site, green line = male frogs from reference sites, yellow line = female frogs from reference sites).

Overall, diversity of OTUs was higher at the contaminated site compared to reference sites; H'= 2.78 and 1.82; D= 307.5 and 208.6, respectively; CF= 0.33 and 103.17; CM= 3.89 and 271; RF= 1.99 and 179; RM= 1.56 and 176.12, Shannon and Chao indices, respectively. When comparing alpha diversity of frogs' microbiome among contaminated and reference sites, no significant difference was observed. However, the contaminated site shows a significantly higher dispersion. Further diversity analysis revealed that this difference was due to disparity between males and female frogs skin microbiome observed in contaminated site. The former showing a more than twofold increase in diversity, compared with the latter.

The results demonstrated that the composition of the frogs' skin communities differed in response to the sampling site, thus being influenced by metal contamination, but also by the frog's gender. Proteobacteria, varying between 96.4 % (CF) and 82.8 % (CM), were the most abundant bacterial phyla in all sites. Firmicutes reached 11.2 % in CM but only 3 % or lower in the other samples. The classes Actinobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, Acidobacteria, Cyanobacteria, Gemmatimonadetes and Thermi were at percentages as low as 0.1 % in all sites. Members of Proteobacteria included mainly Gammaproteobacteria (45.7 %, CM - 95.5 %, CF),

Alphaproteobacteria (0.7 %, CF - 19 %, CM) and Betaproteobacteria (0.1 %, CF - 18 %, CM). Bacilli (0.5 %, CF - 11.0 %, CM) and Clostridia (0.1 %, CM - 2.8%, CF) were the predominant Firmicutes. Moraxellaceae constituted 79.4 % of the total in RF and 69.3 % of the total in RM, while where almost absent at the contaminated site (0 %, CF - 7.9 %, CM). Xanthomonadaceae (belonging to the same species *Stenotrophomonas rhizophila*) constituted 94,8 % of the total in CF but only 2.3 % of the total in CM, while where absent at the reference sites. Enterobacteriaceae was the third most present family (0.2 %, CF - 7.4 %, RM; 12.0 %, RF - 34.0 %, CM) (Fig.6.4).

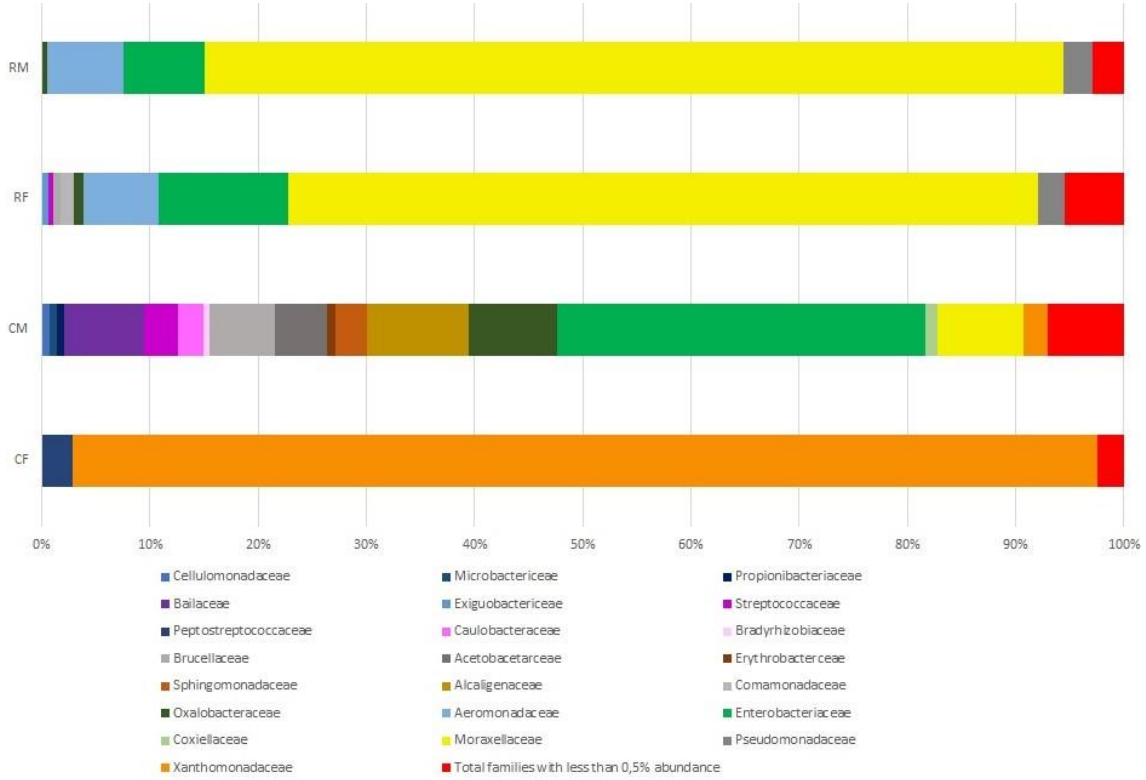


Fig.6.4: Percentage composition of the whole *Pelophylax perezi* skin microbial community at family level as resulting from the DNA barcoding analysis. The chart is divided in rows for male (RM) and female frogs from the reference sites (RF) and for males and females sampled at the metal contaminated site (CM and CF, respectively).

At all diversity levels, the samples coming from male frogs at the impacted site showed the higher diversity, followed by samples from the reference sites (regardless of the frog's gender), while females frogs from the impacted site showed the lower microbiome diversity of all. Because such a high prevalence of sequences from a single species of a single family, in the CF sample, raised doubts, a different approach was also tried. All identical sequences were removed from the calculations and the patterns of diversity were checked again. Nevertheless, the same differentiation between CF and the reference sites (RM and RM) remained; the same can be said for the differences between CF and CM. When the percentages of abundance were recalculated CF was characterized by a prevalence of Peptostreptococcaceae (60.8 %), which were absent in all the other samples; followed by Bacillaceae

(8.7 %, CF - 7.7 % CM; less than 1 % at RF and RM) and Brucellacae (8.6 %, CF – 6.2 % CM; less than 1 % at RF and RM).

PCoA analysis (Fig.6.5), revealed a considerable distance between contaminated and pristine sites, accompanied by a difference between males and females, confirming the results of DGGE.

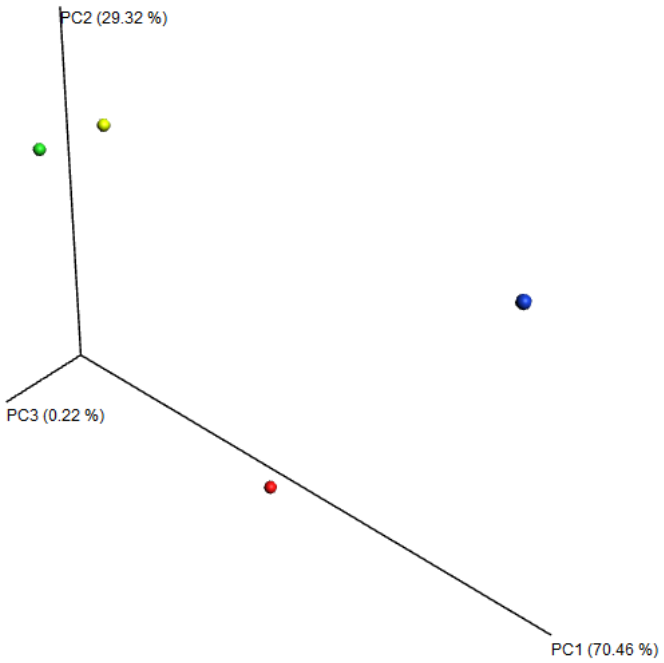


Fig.6.5: PCoA for the total microbiome of the analyzed *Pelophylax perezi* skin bacterial samples (blue dot = male frogs from contaminated site, red dot = female frogs from contaminated site, green dot = male frogs from reference sites, yellow dot = female frogs from reference sites).

Microbiome diversity by cultivable fraction of *P. perezi* skin microbiome:

Cultivable bacteria (CFU) varied in $\log_{10}$, considering all frogs was 5.22±3.86. The CFU values were similar when comparing contaminated (AF) and reference sites (LC, BR1 and BR2): 5.22±4.11 and 5.21±4.18, respectively. When comparing males and female, the former showed less CFU per frog comparing with females, with an average of 4.99±2.88 and 5.94±4.84, respectively. No statistical differences were found between the abundances of the cultivable microbiome on the sampled frogs, independently of the site.

A total of 166 bacterial isolates were obtained in this study to determination of the frogs' skin microbiome composition and further understanding their tolerance to AMD.

Phylogenetic analysis of the 16S rRNA gene sequences of the bacterial isolates from all the sites revealed four phyla within the domain Bacteria: Proteobacteria, Actinobacteria, Firmicutes and Bacillii. Isolates belonging to the phylum Proteobacteria were dominant in all the sites, independently from contamination or frog's gender. AF had 62 % Protebacteria, followed by 20 % Firmicutes, 13 % Actinobacteria and 5 % Bacillii. While LC, BR1 and BR2 had 66 % Proteobacteria, followed by

17.5 % Firmicutes, 17.5 % Actinobacteria but no Bacillii. Females frogs had 64% Proteobacteria, followed by 20 % Firmicutes, 14 % Actinobacteria and 2 % Bacillii. While males had 64.5 % Proteobacteria, followed by 18.8 % Firmicutes, 15.7 % Actinobacteria but the class Bacillii was not observed. Moreover, members of the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria and Bacilli were identified in frog's microbiome by cultivation methods. The most abundant families in the non-contaminated site were Enterobacteriaceae (20.3 %), Microbacteriaceae (14.5 %), Moraxellaceae (14.5 %) and Pseudomonodaceae (14.5 %) while in the contaminated site were Enterobacteriaceae (31.3 %) and Staphylococcaceae (12.5 %) (Fig.6.6).

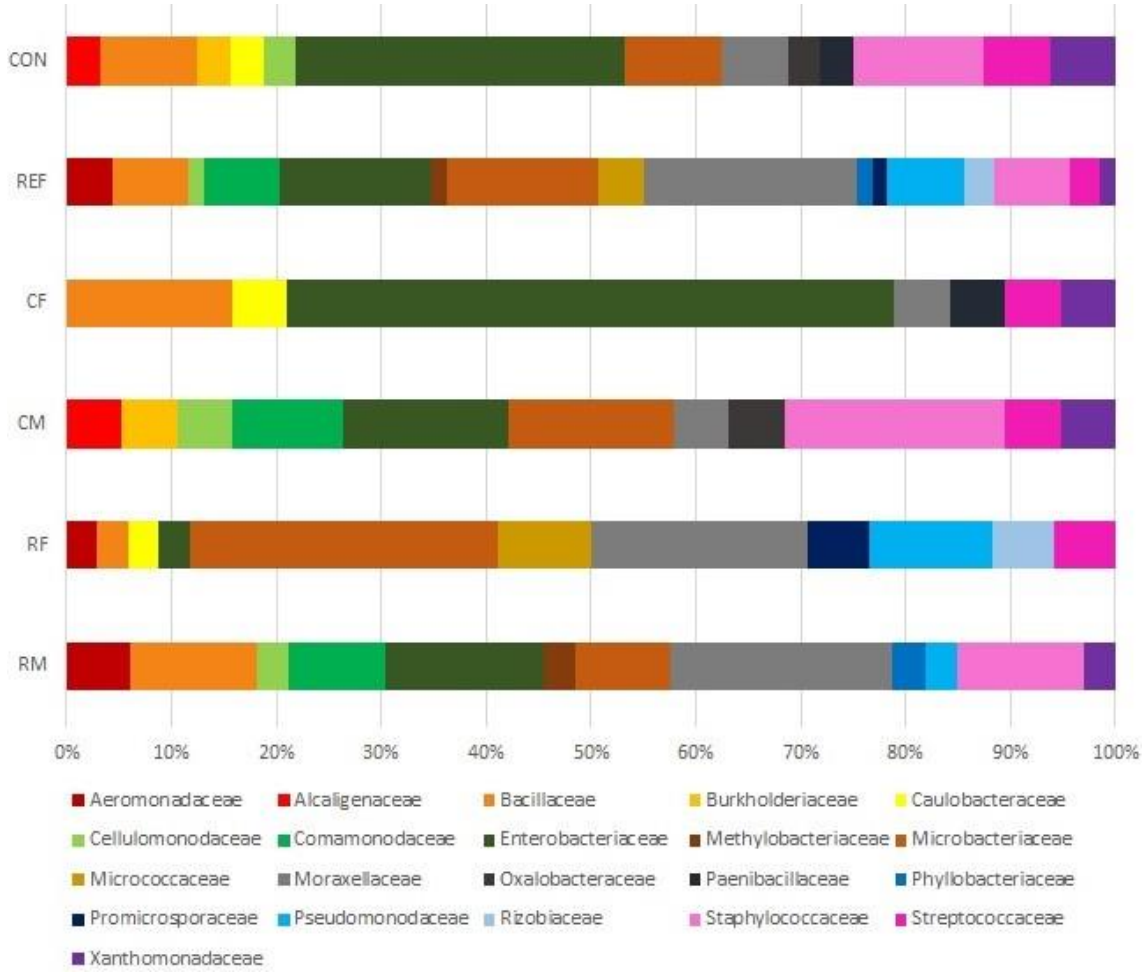


Fig.6.6: Composition, in percentage, of the cultivable fraction of the microbial community (166 isolates), sampled in *Pelophylax perezi* skin, at family level. The chart is divided in rows for total samples from the reference sites (REF), total samples from the metal contaminated site (CON), male (RM) and female (RF) frogs sampled at the reference sites and for males and females sampled at the metal contaminated site (CM and CF, respectively).

The bacterial diversity, based on cultivation methods, was estimated by Shannon–Wiener diversity index (H') and Margalef index (d). H' of the microbial community were: 4.29 for RM, 4.21 for RF; 3.46 for CM, and 3.11 for CF. While d index were 2.48, 2.51, 2.24 and 2.09, respectively.

Skin microbiome of CF counted 7 families only, with Enterobacteriaceae composing 58 % of the community. Skin microbiome of CM counted 11 bacteria families with Staphylococcaceae composing 21.0 %, Microbacteriaceae composing 15.8 % and Enterobacteriaceae composing 15.8 % of the community respectively. Skin microbiome of RF was composed of bacteria counted 11 families, with Microbacteriaceae composing 29.5 % and Moraxellaceae 20.6 % of the total community. The microbiome of RM counted 12 bacterial families, with Moraxellaceae and Enterobacteriaceae, composing 21.2 % and 15.0 %, respectively, were the most abundant.

The skin microbiome of the *P. perezi* showed a core group of bacteria composed by strains of the genera *Acinetobacter, Bacillus, Lactococcus, Microbacterium, Pseudomonas,* and *Stenotrophomonas* (Fig.6.7). Additionally to the core group, males showed exclusively *Cellulomonas, Enterobacter, Serratia* and *Staphylococcus;* while females *Brevundimonas* and *Citrobacter*. Microbial community of the frogs from AF showed exclusively *Bordetella, Cupriavidus, Erwinia, Herbaspirillum* and *Pantoea;* while frogs from LC, BR1 and BR2 showed *Aeromonas* and *Cellulosimicrobium*.

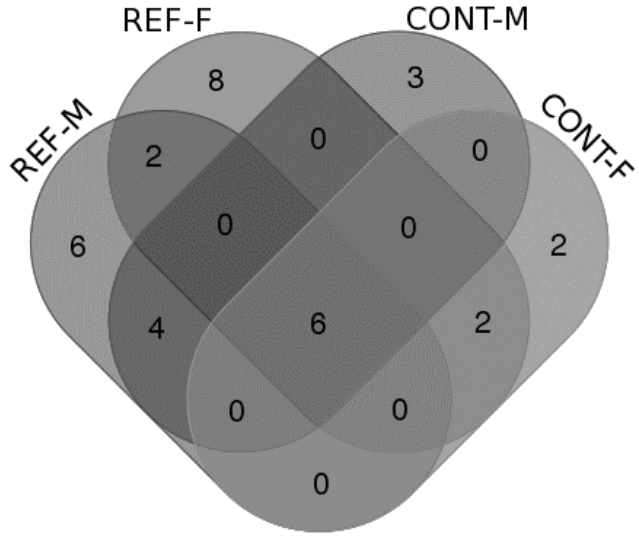


Fig.6.7: Venn diagram of the whole *Pelophylax perezi* skin microbial community. The central part consists in a core group, composed by bacteria belonging to the genera *Acinetobacter, Bacillus, Lactococcus, Microbacterium, Pseudomonas,* and *Stenotrophomonas*; it is possible to note how sixteen strains are present only in the reference sites while just five strains are restricted to the contaminated site.

The RDA analysis, for the cultivable microbiome fraction, supports a strong and clear distinction of the microbial community based on two factors: contamination of the sampling site, and the gender of the frog (Fig.6.8).

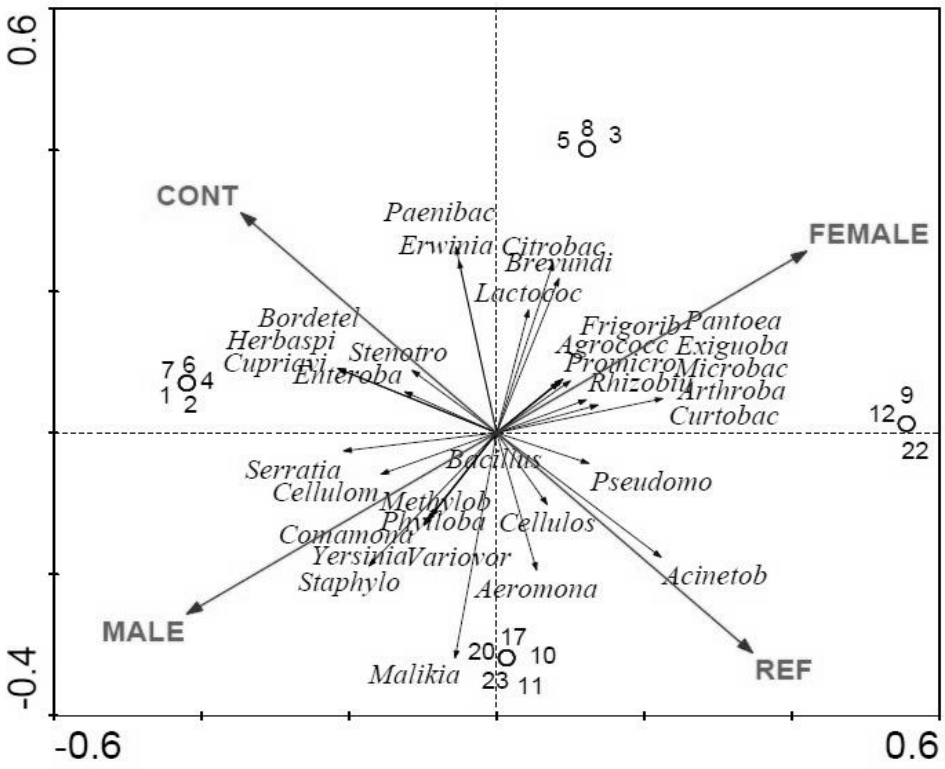


Fig.6.8: RDA analysis of *Pelophylax perezi* skin bacterial samples, based in the differences between metal contaminated and reference sites and between male and female frogs' microbial community.

Tolerance of bacterial strains to AMD:

Statistical analysis revealed a significant inhibitory effect of the acid mine drainage, at 75 % of concentration, on all the cultured bacterial strains (ANOVA, $F_{3\text{-}800}$=11.024, p<0.0001). Final optical density was significantly lower when the strains were exposed to 75 % AMD concentration, while no effect was detected at lower concentrations (Fig.6.9).

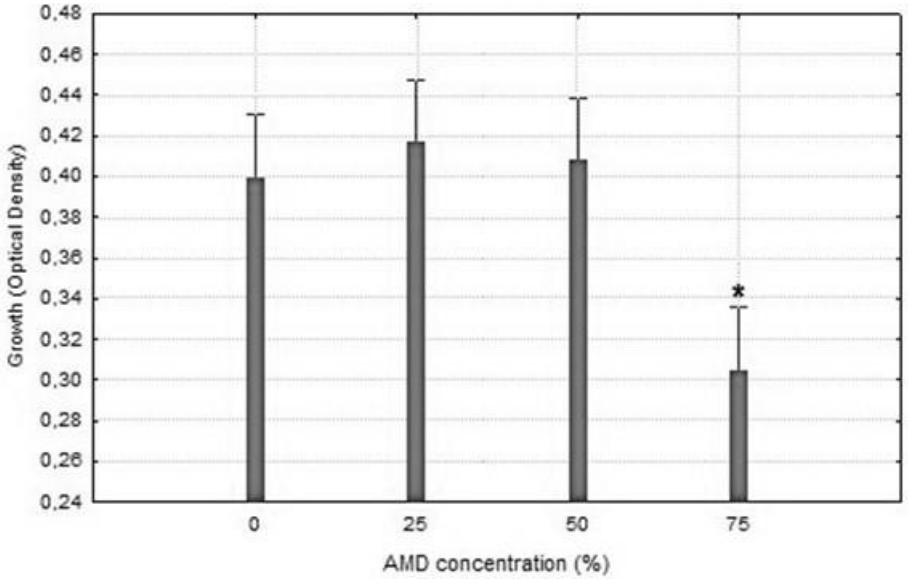


Fig.6.9: Average growth of *Pelophylax perezi* skin microbial community after being exposed to serial dilutions of acid mine drainage. Error bars represent standard deviation.

No significant differences in tolerance to AMD were detected between bacterial strains sampled in contaminated or pristine sites (ANOVA, $F_{1\text{-}800}$=2.6944, p=0.10110), confirmed by the non-metric multidimensional scale (NMDS, stress factor 0.01, Fig.6.10). All the bacteria strains group together, except for some outliers from the contaminated site (*Serratia, Stenotrophomonas, Phyllobacterium* and *Erwinia*). A second NMDS analysis (stress factor 0.01) revealed the same data structure when comparing strains sampled independently of the gender of the frog as a factor influencing tolerance to AMD.

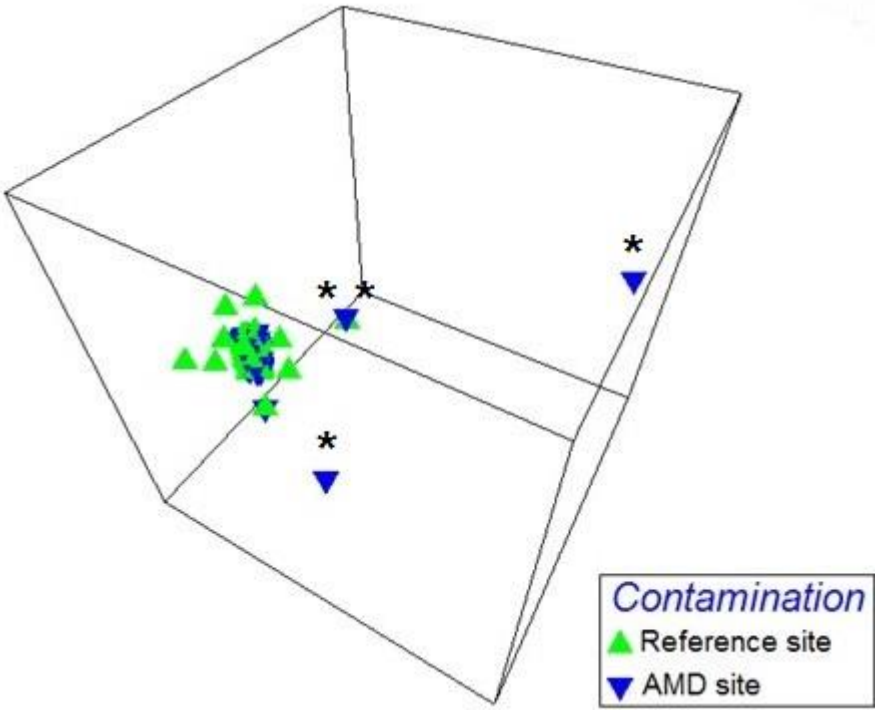


Fig.6.10: NMDS (Non-Metric-Multi-Dimensional-Scaling) analysis discriminating *Pelophylax perezi* skin samples' microbial community's tolerance to acid mine drainage (AMD) by site (green = reference; blue = contaminated); the asterisks denote four strains which are significantly more tolerant to AMD, up to more than 75% concentration.

Due to the appearing outliers, a SIMPER analysis was performed to check if some of the cultured strains revealed a significant tolerance to AMD when compared to the others. Four genera, *Erwinia, Serratia, Stenotrophomonas* and *Phyllobacterium*, all isolated from male frogs, showed higher contribution for bacterial tolerance to 75 % of AMD concentration (cut off percentages for test contributions were: 91.09, 90.14, 94.01 and 90.81, respectively).

Discussion and conclusions

The environment is a key factor on selection of microbes in different habitats, including in frogs' skin microbiome. In the present work, Perez frogs' skin microbial community was studied by cultivation and molecular methods, and it was observed that environment characteristics and gender of the frog influenced its composition. The environment influence on amphibians' skin microbial community was recently reported (Costa *et al.*, 2016) and has been underestimated. The present study shows that, to explore the amphibians' skin microbiome it is not sufficient to analyze it solely on the basis of samples taken from pristine sites. Furthermore, the results also showed how the microbial community was different in males and females of the Perez's frog. This is a novelty that needs to be investigated and confirmed for other frog species. The reason for this unexpected diversification is still unknown. One hypothetical cause could be the possible difference, in mucus's peptides composition between males and females (in turn due to different hormonal inductions) (Edwards, 1979; Boutilier *et al.*, 1992). This difference was evident when analyzing microbiome's average diversity of the whole community. The present study unveiled that the richness, evaluated by 16S rRNA gene-based metagenomics, of *P. perezi* skin microbiome is comparable with the previous study on the same frog species (Costa *et al.*, 2016) and it is in the same order of magnitude than that found for other amphibian species. The present work detected 13 different phyla of bacteria that is comparable to the 16 phyla found on *Rana italica* (Federici *et al.*, 2015), the most related species to the Perez's frogs which was investigated about its skin microbiome. Furthermore, in three different species of frogs from central USA, 10 to 18 different phyla were detected (McKenzie *et al.*, 2012); all these studies relied on Illumina analysis to determine species richness. The bacterial diversity in *P. perezi* skin samples was dominated by Proteobacteria and followed by Actinobacteria and Firmicutes, which is in agreement with findings from previous studies conducted on other amphibian species (Federici *et al.*, 2015; McKenzie *et al.*, 2012; Walke *et al.*, 2014; Kueneman *et al.*, 2014; Costa *et al.*, 2016). The skin microbiome did show significant differences between metal contaminated and reference sites. However, while it was comparable between RF and RM, it was very different between CF and CM. The latter (male frogs from metal contaminated site) showed very high diversity compared with all the other samples. While the diversity of CF was greatly reduced by the almost exclusive prevalence of *Stenotrophomonas rhizophila*. In the eventuality that such a high abundance may result from sample processing, the diversity of CF was re-evaluated excluding *S. rhizophila*; the obtained pattern was still in line with a marked difference between metal contaminated and uncontaminated location. On the other hand, the differences between CF and CM lowered but were still present (at the same order of magnitude of the differences between RF and RM). The present study also evaluated

diversity by cultivable strains method and succeeded into obtaining more cultivable isolates from amphibians' skin than any other study before. The 166 strains isolated, fairly exceed the 40 that were isolated from *Rana muscosa* (Woodhams and Vredenburg, 2007), the 19 isolated from *Platheon cinereus* (Lauer *et al.*, 2008) and even the 74 previously isolated from *P. perezi* (Costa *et al.*, 2016). On the other hand, diversity calculated for the cultivable fraction of the microbiome, highlighted the highest diversity at reference sites. The analysis of cultivable strains revealed how members of the family Pseudomonodaceae are present only in samples from the reference sites. However, Pseudomonodaceae were found by metagenomic analysis, in this study, also at the metal contaminated site. This family is widespread, occurring in almost any habitat associated with freshwater, its members had been encountered onto the skin of different species of amphibians and had been shown the ability to produce compounds which can inhibit bacterial and fungal pathogens growth (Woodhams and Vredenburg, 2007; Harris *et al.*, 2009; Lauer *et al.*, 2008; Rebollar *et al.*, 2016; Federici *et al.*, 2015). On the other hand, members of the family Moraxellaceae were found to grow more abundantly on Perez's frogs sampled at references sites, in the present work (both by Illumina sequencing and cultivation). However, members of both Pseudomonodaceae and Moraxellaceae were found at the same metal contaminated site, on *P. perezi* by Costa et al. (2016). The metal contaminated site from which samples came for this last study, coincide with the location visited in the present work. Thus, suggesting that *P. perezi* skin microbiome could be subject to temporal variations. Furthermore, Moraxellaceae (especially genus *Acinetobacter*) were found more abundant (using Illumina) in individuals of *Rana italica* subject to parasites infestation (Federici *et al.*, 2015). The reason for all the above-discussed differences, and more specifically the presence of Pseudomonodaceae and Moraxellaceae, can be associated with: 1) site-specific conditions; 2) temporal variation in the composition of the skin microbiome (at the same location), as indicated by comparison of the present results with those from Costa *et al.* (2016); 3) to species specificity of the microbial community, as already demonstrated in literature (McKenzie *et al.*, 2012; Kueneman *et al.*, 2014).

The toxicity assays, performed in this work, proved that the isolated bacterial stains growth was significantly lower when they were exposed to 75 % AMD concentration, while no effect was detected at lower concentrations. This result suggests a possible threshold level in AMD tolerance for *P. perezi* microbial communities. Even so, the NMDS analysis shows a compact group, leading to the facts that: 1) microbial communities from all sampling sites harbor both sensitive and tolerant strains to AMD; 2) all strains, being able to grow, at least within 50% AMD, show a possible intrinsic capability of the Perez's frog skin microbiome, to tolerate metal contaminated waters. The SIMPER analysis, detected four strains, which significantly contribute more for tolerance to AMD. Three of

them are known to include opportunistic pathogens species (*Erwinia*, *Serratia* and *Stenotrophomonas*), which are commonly found onto plants or fishes (Skrodenyte-Arbaciauskiene *et al.*, 2006; Wolf *et al.*, 2002). Between them only *Stenotrophomonas* belongs to the Perez's frog core group of cultivable bacteria. While *Erwinia* was encountered only in samples belonging to the contaminated site. *Serratia* was encountered only in CM samples. Which means that, samples from AF had three highly tolerant strains, while samples from LC, BR1 And BR2 only had two: *Stenotrophomonas* and *Phyllobacterium* (among cultivable strains). The latter was isolated from RM, a possible reason for this is that males, tend to be a little less philopatric, going from pond to pond in order to find a possible mate (Smith and Green, 2005; Bowne and Bowers, 2004). Therefore, they can possibly enter in contact with more bacterial strains coming from different environments. Overall, *P. perezi* skin microbiome composition can suggest that Perez's frogs naturally harbor strains which can cope with AMD contamination. If a protective function of the microbiome is true, it can be exerted by different strains. Or it can be either a feature of: 1) a non-cultivable part of the microbial community; 2) the community as a whole, but not of its single components; 3) more strains in the community, acting together or independently.

This study has shown a far more complex degree of differentiation than that which resulted by sampling in pristine sites only. Thus, it is critically important to expand the future research by investigating the effects of various stressors on the skin related microbiome. This study confirms how amphibians skin microbial community is influenced by metal contamination, how it can variate temporally at the same site and how it can be different in males or females (at least in the Perez's frog). This novelty could be crucial in understanding how skin microbiome works in amphibians and how it can be related to frog's immune system capabilities. Because the difference between males and females skin microbiome could unveil particular physiological conditions, also related with different mucus production and composition (Edwards, 1979; Boutilier *et al.*, 1992). What could be the impact of such findings on endangered species, threatened by different kind of pollution or impacted by pathogens, it is still to be discovered. But is certain that environmental pollution highly affect the composition of the microbial community found onto amphibians' skin. If metal contamination can significantly impact amphibians' skin bacterial community. This can potentially increase the risk and virulence of widespread diseases, like *Batrachochytrium dendrobatidis*. Therefore, further research is needed to explore the interaction of pollutants with amphibians' skin microbial community; moreover, differences between male and females should be investigated for other amphibian species.

Acknowledgements

This work was supported by FEDER funds within the PT2020 Partnership Agreement and Compete 2020- Programa Operacional Factores de Competitividade, by the Portuguese Foundation for Science and Technology (FCT), by the CESAM and CEMUC strategic programme (UID/AMB/50017/2013 and UID/EMS/00285/2013, respectively) and the research project GENEROSI project number PTDC/BIA-BIC/3488/2012. This work was also funded by national funds via FCT/MEC (PIDDAC) by the doctoral fellowship of Emanuele Fasola SFRH/BD/88955/2012, by postdoctoral fellowship of Diogo Neves Proença SFRH/BPD/100721/2014 and by investigator FCT grant of Isabel Lopes IF/00475/2013. The authors would also like to thank Tania Duarte, Catarina Coelho and Pedro Farias for their help in the laboratory.

References

ALTSCHUL, S F; MADDEN, T L; SCHAFFER, A A; ZHANG, J; ZHANG, Z; MILLER, W; LIPMAN, D J - Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic acids research**. ISSN 0305-1048. 25:17 (1997) 3389–3402.

BALÁŽ, Vojtech; VOROS, Judit; CIVIS, Petr; VOJAR, Jiri; HETTYEY, Attila; SOS, Endre; DANKOVICS, Róbert; JEHLE, Robert; CHRISTIANSEN, Ditte G; CLARE, Frances; FISHER, Matthew C; GARNER, Trenton W J; BIELBY, Jon - Assessing Risk and Guidance on Monitoring of Batrachochytrium dendrobatidis in Europe through Identification of Taxonomic Selectivity of Infection. **Conservation biology : the journal of the Society for Conservation Biology**. ISSN 1523-1739. 0:0 (2013) 1–11. doi: 10.1111/cobi.12128.

BECKER, Matthew H.; BRUCKER, Robert M; CHRSTIAN, R; HARRIS, Reid N; MIMBIOLE, Kevin P C; SCHWANTES, Christian R - The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus. **Applied and Environmental Microbiology**. 75:21 (2009) 6635–6638. doi: 10.1128/AEM.01294-09.

BELDEN, Lisa K.; HUGHEY, Myra C.; REBOLLAR, Eria A.; UMILE, Thomas P.; LOFTUS, Stephen C.; BURZYINSKI, Elizabeth A.; MINBIOLE, Kevin P C; HOUSE, Leanna L.; JENSEN, Roderick V.; BECKER, Matthew H.; WALKE, Jenifer B.; MEDINA, Daniel; IBANEZ, Roberto; HARRIS, Reid N. - Panamanian frog species host unique skin bacterial communities. **Frontiers in Microbiology**. ISSN 1664302X. 6:OCT (2015) 1–21. doi: 10.3389/fmicb.2015.01171.

BLAUSTEIN, A. R.; HOKIT, D. G.; O'HARA, R. K. - Pathogenic fungus contributes to amphibian losses in the Pacific Northwest. **Biological Conservation**. 67 (1994) 251–254.

BLAUSTEIN, AR; WAKE, DB; SOUSA, WP - Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. **Conservation Biology**. (1994).

BOUTILIER, R. G.; STIFFLER, D. F.; TOEWS, D. - Exchange of respiratory gases, ions and water in amphibious and aquatic amphibians. In BURGGRENWW (Ed.) - **Environmental Physiology of the Amphibians.** London : University

of Chicago Press: Chicago, 1992. p. 81–124.

BOWNE, DR; BOWERS, MA - Interpatch movements in spatially structured populations: a literature review. **Landscape Ecology**. (2004) 1–20.

BREM, F.; MENDELSON, J. R.; LIPS, K. R. - Field-sampling protocol for *Batrachochytrium dendrobatidis* from living amphibians, using alcohol preserved swabs. [On line] Available at :http//www.amphibians.org>.

BRESCIANO, JC; SALVADOR, CA; PAZ-Y-MINO, C; PAROD-MERINO, AM; BOSH, J; WOODHAMS, DC - Variation in the Presence of Anti-*Batrachochytrium dendrobatidis* Bacteria of Amphibians Across Life Stages and Elevations in Ecuador. **EcoHealth**. 12:2 (2015) 310–319. doi: 10.1007/s10393-015-1010-y.

BRINK, Paul J. VAN DEN; BRAAK, Cajo J. F. - Principal response curves: Analysis of time-dependent multivariate responses of biological community to stress. **Environmental Toxicology and Chemistry**. ISSN 1552-8618. 18:2 (1999) 138–148. doi: 10.1002/etc.5620180207.

CAPORASO, J.Gregory; KUCZYNSKI, Justin; STOMBAUGH, Jesse; BITTINGER, Kyle; BUSHMAN, Frederic D; COSTELLO, Elizabeth K; FIERER, Noah; PENA, Antonio Gonzalez; GOODRICH, Julia K; GORDON, Jeffrey I; HUTTLEY, Gavin A; KELLEY, Scott T; KNIGHTS, Dan; Koenig, JEREMY E; Ley, RUTH E; LOZUPONE, Catherine A; McDONALD, Daniel; MUEGGE, Brian D; PIRRUNG, Meg; REEDER, Jens; SEVINSKY, Joel R; TURNBAUGH, Peter J; WALTERS, William A; WIDMANN, Jeremy; YATSUNENKO, Tanya; ZANEVELD, Jesse; KNIGHT, Rob - QIIME allows analysis of high-throughput community sequencing data. **Nature methods**. ISSN 1548-7091. 7:5 (2010) 335–336. doi: 10.1038/nmeth.f.303.

COSTA, S.; NEVES PROENÇA, D.; RIBEIRO, R; LOPES, I; VASCONCELO MORAIS, P - Diversity of cutaneous bacterial community of Pelophylax perezi populations inhabiting different environments. **Science of the Total Environment**. ISSN 1098-6596. in press (2016). doi: 10.1016/j.scitotenv.2016.07.230.

DESANTIS, T. Z.; HUGENHOLTZ, P.; LARSEN, N.; ROJAS, M.; BRODIE, E. L.; KELLER, K.; HUBER, T.; DALEVI, D.; HU, P.; ANDERSEN, G. L. - Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. **Applied and Environmental Microbiology**. ISSN 00992240. 72:7 (2006) 5069–5072. doi: 10.1128/AEM.03006-05.

DOBSON, Alleson; COTTER, Paul D.; ROSS, R.; HILL, Colin - Bacteriocin production: A probiotic trait? **Applied and Environmental Microbiology**. ISSN 00992240. 78:1 (2012) 1–6. doi: 10.1128/AEM.05576-11.

EDWARDS, H. A. - A novel mechanism for salt and fluid transport across epithelia. **Journal of experimental biology.** 83 (1979) 335–338.

FASOLA, E.; RIBEIRO, R.; LOPES, I. - Microevolution due to pollution in amphibians: A review on the genetic erosion hypothesis. **Environmental Pollution**. ISSN 18736424 02697491. 204:2015). doi: 10.1016/j.envpol.2015.04.027.

FEDERICI, Ermanno; ROSSI, Roberta; FIDATI, Laura; PARACUCCHI, Romina; SCARGETTA, Silvia; MONTALBANI, Elena; FRANZETTI, Andrea; LA PORTA, Gianandrea; FAGOTTI, Anna; SIMONCELLI, Francesca; CENCI, Giovanni; DI ROSA, Ines - Characterization of the Skin Microbiota in Italian Stream Frogs (*Rana italica*) Infected and Uninfected by a Cutaneous Parasitic Disease. **Microbes and Environments**. ISSN 1347-4405. 30:3 (2015) 262–269. doi: 10.1264/jsme2.ME15041.

FERNÁNDEZ-BENÉITEZ, María José; ORTIZ-SANTALIETRA, Manuel Eloy; LIZANA, Miguel; DIEGUEZ-URIBEONDO, Javier - Differences in susceptibility to *Saprolegnia* infections among embryonic stages of two anuran species. **Oecologia**. . ISSN 1432-1939. 165:3 (2011) 819–26. doi: 10.1007/s00442-010-1889-5.

HAMMER, Øyvind; HARPER, David A. T.; RYAN, Paul D. - Past: Paleontological Statistics Software Package for Education and Data Analysis. **Palaeontologia Electronica**. 4:1 (2001) 1–9.

HARRIS, RN; BRUCKER, RM; WALKE, JB - Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. **The ISME Journal**. ISSN 1751-7362. 3:7 (2009) 818–824. doi: 10.1038/ismej.2009.27.

KIM, Ok-Sun; CHO, Yong-Joon; LEE, Kihyun; YOON, Seok-Hwan; KIM, Mincheol; NA, Hyunsoo; PARK, Sang-Cheol; JEON, Yoon Seong; LEE, Jae-Hak; YI, Hana; WON, Sungho; CHUN, Jongsik - Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. **International journal of systematic and evolutionary microbiology**. ISSN 1466-5034. 62:Pt 3 (2012) 716–721. doi: 10.1099/ijs.0.038075-0.

KLINDWORTH, Anna; PRUESSE, Elmar; SCHWEER, Timmy; QUAST, Christian; HORN, Matthias; GLOCKNER, Frank Oliver - Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. **Nucleic Acids Research**. ISSN 03051048. 41:1 (2013) 1–11. doi: 10.1093/nar/gks808.

KUENEMAN, J. C.; PARFREY, L.W.; WOODHAMS, Douglas C.; ARCHER, H.M.; McKENZIE, R.; KNIGHT, V.J. - The amphibian skin-associated microbiome across species, space and life history stages. **Molecular ecology**. 23 (2014) 1238–1250.

LAUER, A. - Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. **The ISME Journal** 2 (2008) 145–157. doi: 10.1038/ismej.2007.110.

LAUGEN, Ane T.; LAURILA, Anssi; MERILÄ, Juha. - Maternal and genetic contributions to geographical variation in *Rana temporaria* larval life-history traits. **Biological Journal of the Linnean Society**. ISSN 00244066. 76:1 (2002) 61–70. doi: 10.1111/j.1095-8312.2002.tb01714.x.

LINDGREEN, Stinus - AdapterRemoval: easy cleaning of next-generation sequencing reads. **BMC research notes**. ISSN 1756-0500. 51:2012) 337.

LONGO, Ana V; ZAMUDIO, Kelly R. - Environmental fluctuations and host skin bacteria shift survival advantage between frogs and their fungal pathogen. **The ISME Journal**. ISSN 1751-7362. 2016) 1–13. doi: 10.1038/ismej.2016.138.

LUDWIG, Wolfgang; STRUNK, Oliver; SCHLEIFER, Karl-Heinz - ARB: a software environment for sequence data. **Nucleic acids research**. ISSN 1362-4962. 32:4 (2004) 1363–1371. doi: 10.1093/nar/gkh293.

MCKENZIE, V. J.; LUDWIG, Wolfgang; STRUNK, Oliver; SCHLEIFER, Karl-Heinz - Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. **The ISME Journal**. (2012) 588–596. doi: 10.1038/ismej.2011.129.

MCKENZIE, VJ; PETERSON, AC - Pathogen pollution and the emergence of a deadly amphibian pathogen. **Molecular ecology**. (2012) 5151–5154.

MUYZER, G.; WAAL, E. C. DE; UITTERLINDEN, A G. - Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. **Applied and environmental microbiology**. ISSN 0099-2240. 59:3 (1993) 695–700.

OKSANEN, Jari; KINDT, Roeland; LEGENDRE, Pierre; O'HARA, Bob; SIMPSON, Gavin L.; SOLYMOS, Peter; STEVENS, Henry H.; WAGNER, Helene - The vegan Package. (2016) 285.

PASK, JD - The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. **Global Change. (**2012) 1231–1238. doi: 10.1111/j.1365-2486.2011.02622.x.

PEARMAN, P. B.; GARNER, T. W. J. - Susceptibility of Italian agile frog populations to an emerging Ranavirus parallels population genetic diversity. **Ecology Letters**. 8 (2005) 401–408.

PITCHER, D.G; N. A.Saunders; R. J.Owen - Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. **Letters in Applied Microbiology**. 8:4 (1989) 151–156.

POUNDS, J.Alan; BUSTAMANTE, Martín R; YOUNG, Bruce E - Widespread amphibian extinctions from epidemic

disease driven by global warming. **Nature**. ISSN 1476-4687. 439:7073 (2006) 161–7. doi: 10.1038/nature04246.

PRUESSE, Elmar; PEPLIES, Jörg; GLÖCKNER, Frank Oliver - SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. **Bioinformatics (Oxford, England)**. ISSN 1367-4811. 28:14 (2012) 1823–1829. doi: 10.1093/bioinformatics/bts252.

RAINEY, F. A.; WARD-RAINEY, N., KROPPENSTEDT, R. M.; STACKEBRADT, E. - The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of Nocardiopsaceae fam. nov. **International journal of systematic bacteriology**. ISSN 0020-7713. 46:4 (1996) 1088–1092. doi: 10.1099/00207713-46-4-1088.

RAVEN, P. H.; JOHNSON, G. B. - **Biology**. 10th. ed. [S.l.] : McGraw-Hill, 2014

REBOLLAR, Eria; HUGHHEY, Myra C; BELDEN, Lisa K - Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. **The ISME journal**. ISSN 1751-7370. 2016) 1–14. doi: 10.1038/ismej.2015.234.

SALUNKE, B. K. SALUNKE, R. C.; PATOLE, M. S.; SHOUCHE, Y. S. - *Wolbachia* and termite association: Present status and future implications. **Journal of Biosciences**. ISSN 02505991. 35:2 (2010) 171–175. doi: 10.1007/s12038-010-0020-8.

SCHMIEDER, Robert; EDWARDS, Robert - Fast identification and removal of sequence contamination from genomic and metagenomic datasets. **PLoS ONE**. ISSN 19326203. 6:3 (2011) e17288. doi: 10.1371/journal.pone.0017288.

SKRODENYTE-ARBACIAUSKIENE, V.; SRUOGA, A.; BUTKAUSKAS, D. - Assessment of microbial diversity in the river trout Salmo trutta fario L. intestinal tract identified by partial 16S rRNA gene sequence analysis. **Fisheries Science**. 72 (2006) 597–602. doi: 10.1111/j.1444-2906.2006.01189.x.

SMIBERT RM & KRIEG NR - **Phenotypic characterization. Methods for General and Molecular Bacteriology**. [S.l.] : American Society for Microbiology, Washington, DC., (1994).

SMITH, M.Alex; GREEN, David M. - Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? **Ecography**. 28:1 (2005) 110–128.

STAMATAKIS, A. - RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. **Bioinformatics**. . ISSN 1367-4803. 22:21 (2006) 2688–2690. doi: 10.1093/bioinformatics/btl446.

STEVENSON, LA; ALFORD, RA; BELL, SC; ROZNIK, EA - Variation in thermal performance of a widespread pathogen, the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. **PloS one**. 8:9 (2013) 1–14. doi: 10.1371/journal.pone.0073830.

WALKE, J.B.; BECKER, Matthew H.; BELDEN, L.K. - Amphibian skin may select for rare environmental microbes. **The ISME Journal.** 8:2014) 2207–2217.

WOLF, Arite; FRITZE, Antje; HAGEMANN, Martin; BERG, Gabriele - *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. **International Journal of Systematic and Evolutionary Microbiology**. ISSN 14665026. 52:6 (2002) 1937–1944. doi: 10.1099/ijs.0.02135-0.

WOODHAMS, DC; VREDENBURG, VT - Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. **Biological Conservation.** 8 (2007). doi: 10.1016/j.biocon.2007.05.004.

WOODHAMS, Douglas C.; BRANDT, Hannelore; McKENZIE, Valerie - Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. **PLoS ONE**. ISSN 19326203. 9:4 (2014). doi: 10.1371/journal.pone.0096375.

WOODHAMS, Douglas C.; BLETZ, Molly; KUENEMAN, Jordan; McKEnzie, Valerie - Managing Amphibian Disease with Skin Microbiota. **Trends in Microbiology**. ISSN 0966842X. 24:3 (2016) 161–164. doi: 10.1016/j.tim.2015.12.010.

CHAPTER 7.

General discussion and conclusion.

General discussion and conclusions

Amphibians are facing a global decline and pollution is one of the factors driving this process (Allentoft and O'Brien, 2010; Carey and Bryant, 1995; O'Brien and Allentoft, 2010; Vertucci and Corn, 1996). Contaminant-induced genetic erosion can be responsible for the decrease of their populations' genetic variability (Fasola *et al.*, 2015; Medina *et al*., 2007; Ribeiro and Lopes, 2013; Van Straalen and Timmermans, 2002). Many amphibian species are characterized by fragmented and endangered populations (Beebee, 2005; Sparling *et al.*, 2010), which often occur as highly inbred, retaining low genetic variability (Beebee, 2005). Therefore, amphibians' populations could be used as a case study to measure the impacts of contaminant-induced decrease of genetic diversity (contaminant-driven genetic erosion) (Fasola *et al.*, 2015). Among the various pollution sources, the one deriving from mining activity can cause a big impact on the surrounding natural habitats (Adlassnig *et al.*, 2013; Alpers *et al*., 2003; Blowes *et al.*, 2003; Edmondson, 1989; Martins *et al.*, 2009; Nordstrom and Alpers, 1999; Pereira *et al*., 2004; Reglero *et al.*, 2008). Mining-derived metal pollution has been noteworthy in the Iberian Peninsula, especially in the Iberian pyrite belt region (Baranda, 1994; Edmondson, 1989; Higueras *et al.*, 2006; Lindberg *et al.*, 1978; Pereira *et al*., 2004; Reglero *et al.*, 2008). Thus, is important to investigate: 1) how mechanisms of tolerance to metals (both facing chronic or acute exposure) work in amphibians, 2) how genetically determined tolerance mechanisms are inherited, 3) if amphibians' populations can acclimate to metal contamination, 4) if also amphibians skin microbiome can be affected by metal contamination. In this work, these topics were addressed in different life stages: eggs (1, 2), tadpoles (3) and adults (4) of the Perez's frog (*Pelophylax perezi*).

Genetically determined tolerance to metal exposure was evaluated, in *P. perezi* egg masses, in light of the recessive tolerance inheritance (working-) hypothesis (Ribeiro and Lopes, 2013); to investigate if the loss of genetic variability is irreversible after the occurrence of contaminant-driven elimination of alleles in a population. The acquisition of lethal tolerance to metal contamination was evaluated in *P. perezi* tadpoles inhabiting populations historically exposed to metal-rich acid mining effluents. Furthermore, because the increasing research on the important protective role of amphibians' skin microbiome (Becker *et al.*, 2009; Dobson *et al.*, 2012; Harris *et al*., 2009; Pask, 2012; Woodhams *et al.*, 2016), its diversity and capacity to tolerate metal contamination was as well investigated; by the skin microbiome of adults *P. perezi*, inhabiting metal contaminated and reference sites.

In this context, the following highlights can be derived from the research performed in the present work:

- ***Amphibians populations could be ideal case studies for contaminant-driven genetic erosion, but their dispersing capabilities should be taken into account when sampling metapopulations:***

Amphibians populations could be used as models to measure the impacts of contaminant-driven genetic erosion. However, genetic erosion in amphibians population is still debated, due to contrasting results in previous studies (Fasola *et al*., 2015). A possible reason could lay in amphibians population structure. Many researchers supposed limited dispersal (causing isolation) as the reason for the metapopulation structures observed in many amphibians species. However, this a priori assumption is often not correct (especially for anurans) (Smith and Green, 2005). Therefore, there is the possibility that past studies could be biased: instead of comparing two different populations, by assuming limited dispersal, they could actually sample the same one; this would impede to correctly evaluate genetic diversity between demes.

- ***The recorded, between egg masses, tolerance variability suggests that tolerance to metal contamination, in P. perezi is genetically determined:***

The low mortality of the controls eggs, in the present work suggests that intrinsic mortality rate (under optimal environmental conditions) of *P. perezi* egg masses, in the field, is very low or even zero. Therefore, mortality in the field could be assumed to be the result of environmental perturbations (for example contamination, predation or other environmental pressures).

In controlled laboratory conditions there was high variability, between egg masses, in the tolerance to AMD or copper. These responses, following exposure to metal contamination, did not variate in function of pH or conductivity. The observed variability was neither function of the environmental conditions at the moment of the egg mass spawning (no correlation was found between tolerance to metal exposure and the recorded environmental variables). Therefore, it is assumed that the observed variability, in tolerance to AMD or copper, could be either: 1) stochastic or 2) genetically determined. Because of the huge variability in egg mass tolerance, covering over two orders of magnitude, the first option can be safely dismissed; thus, the observed variability can be assumed as being due to genetically determined tolerance mechanisms.

- ***Incomplete dominance is the most likely inheritance mechanism of tolerance toward acid mine drainage or copper in the Perez's frog:***

Pelophylax perezi egg masses, exposed to acid mine drainage (AMD) or copper, showed egg tolerance to be distributed over a large range. Tolerance had, in most of the cases a unimodal distribution. Most egg masses, regardless of their tolerance to metals, presented intermediate relative spread values. With no clear association between relative spread and tolerance. This picture supports incomplete dominance as the most likely inheritance mechanism of tolerance to AMD or copper in the Perez's frog, thus excluding dominance, recessivity, underdominance, or overdominance. The analysis performed in this work was based on the hypothesis of a genetic system of a single gene with two alleles, but the conclusions that were drawn are still valid (for the trait as a whole) if implicated genes have more than two alleles, despite the fact that alleles loss probability may be lower. In this scenario, a strong enough metal contamination would eliminate the most sensitive genotypes, but would not result in the fixation of the allele conferring tolerance. This is true also if the genetic basis of tolerance is polygenic. Allele fixation could happen just if the contamination pulse proved almost lethal, then only the most tolerant (homozygous) genotypes would survive. In the same context and using the same methods described previously, The same outcome was observed, for tolerance to copper, between sampling at two reproductive seasons: 2014 and 2016 showing temporal consistency. The worst-case scenario foreseen by the recessive tolerance inheritance hypothesis is alleles fixation, this would considerably lower the genetic variability in a population impacted by (metal) contamination. Such a situation could happen if the genetically determined tolerance is due to (full) recessivity (Ribeiro and Lopes, 2013). In this case, even a not substantially lethal metal input would eliminate the most sensitive genotypes. The dominant allele, not codifying for tolerance, would be eradicated from the population. This scenario would be real in the case that all *P. perezi* egg masses would be either: 1) sensitive (with small relative spread), could result from one homozygous dominant frog mating with any other frog; 2) tolerant (with a small relative spread), from the mating of two homozygous recessive frogs; 3) result of the mating between a heterozygous with either a homozygous recessive or another heterozygous frog, regardless of the egg mass level of tolerance. Furthermore, this (full) recessivity scenario could be true only if relative spread of intermediately tolerant egg masses is higher than those of both sensitive and tolerant ones. The obtained results did not match this situation, not for AMD and neither for copper exposure (in the majority of the tested egg massess), no inverted U bell-shaped association of relative spread versus tolerance could be found. Therefore, only a severe metal contamination should be able to reduce *P. perezi* population's genetic variability at the point to reach alleles fixation.

- ***AMD and copper tolerance could be possibly due to different mechanisms; pH and intensity of the metal contamination could influence the outcome:***

There was a tendency for median lethal time values, for egg masses exposed to copper, to be higher than for those exposed to AMD, and this was consistent between two different years (2014, 2016) in the same populations. It is possible that tolerance to acid mine drainage (a mixture o metals) may involve different physiological mechanisms than those required to cope against copper ions alone. An important determining factor could be the different pH of the effluent compared to that of the copper solution (AMD solution was acid, pH≈3, while copper solutions were neutral pH≈7). Tolerance to different metals ions is likely due to the same response, while the stress given by acidity could be regulated by a different mechanism. Furthermore, increasing pH levels can decrease copper ions absorption, as shown by the biotic-ligand-model, thus lowering copper toxicity (Di Toro *et al*., 2001). As the acid mine drainage contains different metal ions rather than copper alone (Sobral *et al*., 2013), its toxicity can be also the effect of ions, other than copper, or the complex interaction between various metals. The intensity of the metal contamination could also be involved in this scenario, in the present work copper would provoke lethality at a lesser extent than AMD. Some egg masses, exposed to copper (12.5%) showed bimodal patterns which could not be explained by incomplete dominance and which could suggest poligeny. This makes sense in the light of the mentioned difference in median lethal time values, between AMD and copper. Previous research suggests that when a contaminant impact is milder (copper), tolerance tends to have a polygenic basis, possibly involving many minor genes. On the other hand, when a contaminant causes higher lethality (AMD), few genes are responsible for genetically determined tolerance (Hoffmann and Parsons, 1991; Macnair 1991, 1997).

- ***Perez's frog tadpoles, sampled in historically metal impacted sites, do not show high oxidative stress, nor higher tolerance, compared to tadpoles coming from reference sites:***

In the present work, selected biochemical markers of oxidative stress responses (glutathione peroxidase, superoxide dismutase and malondialdehyde) were analyzed in *P. perezi* tadpoles, to assess the enzymatic activity facing the presence of metal-induced rise of reactive oxygen species. Oxidative stress was not higher in individuals sampled at historically metal contaminated sites, compared to those sampled at reference sites. The biochemical markers, obtained for tadpoles in the toxicity assays, also revealed that individuals of Perez's frog, exposed to Pb in the laboratory (10.5 mg/L), did not show oxidative stress (independently from the site of provenience). The lack of increase in oxidative stress, for tadpoles coming from historically metal exposed populations, could imply that tadpoles, inhabiting metal contaminated sites, may produce higher basal constitutive levels

of metallothioneins, this situation can possibly be derived from local adaptation or epigenetics, that allow the organisms to cope with metal contamination.

- ***Metal body burden proved to be a reliable indicator of metal exposure in P. perezi tadpoles, both for those historically-exposed in the field and for the ones exposed in the laboratory:***

Metal body burden was clearly higher for tadpoles historically exposed to metal contamination in the field. Mercury levels, in the sampled tadpoles, were significantly higher at the mercury contaminated sites and lead levels were higher at the historically lead contaminated sites. Thus, confirming that amphibians' populations sampled at metal impacted sites are indeed exposed to mercury and lead contamination due to historical mining activities. Lead body burden data, following the acute laboratory exposure (10.5 mg/L Pb), showed that lead was significantly bioavailable for the exposed tadpoles, independently from their provenience, being in line with the results of previous studies, (Borković-Mitić et al., 2016; Burger and Snodgrass, 2001; Marques et al., 2011). Additionally, tadpoles collected at metal contaminated sites were able to lower their Pb body burden levels (practically by a half) after just one week of mantainance in laboratory conditions (as shown by Pb levels in lab-controls); this result is in line with the decrease of cadmium encountered by Dobrovoljc *et al*. (2003) in tadpoles of *Rana dalmatina*, previously exposed to cadmium and later allowed to eliminate it, in clean laboratory conditions (50% reduction in one week) (Dobrovoljc *et al*., 2003). This could point to the presence of a similarly efficient lead excretion mechanism in the Perez's frog. Perez's frog tadpoles are likely to be affected by metal contamination in the field; because mercury and lead were readily bioavailable, following both acute or chronic exposure. However, this outcome also points out that *P. perezi* can be used as a model organism to assess both acute and chronic exposure to mercury and lead.

- ***No differential tolerance to metal contamination was observed between tadpoles, inhabiting historical metal contaminated sites, compared with those sampled at reference sites, after acute exposure in the laboratory (both metals did not reveal site-dependent differences):***

The present work shows that acute exposure to mercury rapidly induce mortality in Perez's frog tadpoles, being lethal to all tested individuals (from all sampled populations). The exposed tadpoles reacted without detectable site-dependent differences (individuals coming from historically metal contaminated sites did not show higher tolerance than tadpoles sampled at reference sites). Therefore, an acute mercury contamination event, could cause high mortality, independently from previous exposure conditions. On the other hand, acute exposure to lead did not result lethal. It is possible that

P. perezi is very tolerant to lead contamination. However, tadpoles from historically contaminated sites did not proved to be more tolerant than those from reference sites, because no differences in lethal or biochemical responses were found; comparing tadpoles either from reference or historically metal contaminated sites.

- ***Metal contamination influence richness and the composition of P. perezi skin microbiome, the same is true for the differences between male and female frogs:***

Confirming the results obtained in a past study (Costa *et al.*, 2016), this work shows the influence of chemical contamination on amphibians' skin microbial community. The composition of the microbial community was markedly different, both in terms of taxa and their relative abundance, between metal impacted and reference sites. Most previous studies, focused only in pristine areas (McKenzie *et al.*, 2012) but the current work proved that their findings are not sufficient to investigate amphibians skin microbiome. Furthermore, the obtained results also showed a different composition in the microbial community of males and females of *P. perezi*. This is a novelty that had never been found nor investigated in other species. Nevertheless, it could be important because this difference, between males' and females' microbiome, may reveal physiological conditions related with different mucus production and composition. Indeed, a reason for this diversification could relate to mucus's peptides composition, differentiated between males and females (in turn caused by different hormones), this would be an interesting hypothesis to be tested in future research.

- ***High concentrations of AMD inhibit the growth of almost all the cultivable fraction of the skin microbiome:***

The growth of bacterial stains, isolated from the skin of *P. perezi*, was significantly inhibited with exposure to 75% AMD concentration, while no inhibition occurred at lower concentrations. This could be related to a possible threshold level in AMD tolerance for the *P. perezi* microbiome. The obtained results indicate that: a) previous exposition to lower AMD concentration does not cause acclimation of the isolated bacterial strains to higher concentrations; b) all isolated strains were able to grow at 50% AMD concentration; c) all but four isolates were inhibited by 50% AMD concentration. The more resistant strains belonged to the genera: *Erwinia, Phyllobacterium, Serratia* and *Stenotrophomonas*. Three of them are known to include opportunistic pathogens species (*Erwinia, Serratia and Stenotrophomonas*), which are commonly found onto plants or fishes (Skrodenyte-Arbaciauskiene et al., 2006; Wolf et al., 2002), the present work is the first report of such bacterial species on amphibian species. Metal contamination can significantly alter amphibians'

skin bacterial community. This can potentially increase the risk and virulence of widespread diseases, like *Batrachochytrium dendrobatidis*. Therefore, further research is needed to explore the interaction of pollutants with amphibians' skin microbial community.

References

ADLASSNIG, Wolfram *et al.* - Amphibians in metal-contaminated habitats. **Salamandra**. 49:3 (2013) 149–158.

ALLENTOFT, ME; O'BRIEN, J. - Global amphibian declines, loss of genetic diversity and fitness: a review. **Diversity**. 2010).

ALPERS, C. N.; NORDSTROM, D. K.; SPITZLEY, J. - Extreme acid mine drainage from a pyritic massive sulfide deposit: The Iron Mountain end-member. Em JAMBOR, J. L.; BLOWES, D. W.; RITCHIE, A. I. M. (Eds.) - **Environmental Aspects of Mine Wastes.** [S.l.] : Mineralogical Association of Canada Short Course Volume 31, 2003. p. 407–430.

BARANDA, Borja Sainz - The Horcajo mines. **Mineralogical Record**. 25:1 (1994) 1–21.

BECKER, Matthew H. *et al.* - The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus. **Applied and Environmental Microbiology**. 75:21 (2009) 6635–6638. doi: 10.1128/AEM.01294-09.

BEEBEE, T. J. C. - Conservation genetics of amphibians. **Heredity**. ISSN 0018-067X. 95:6 (2005) 423–7. doi: 10.1038/sj.hdy.6800736.

BLOWES, D. W. *et al.* - The geochemistry of acid mine drainage. Em LOLLAR, B. S. (Ed.) - **Environmental Geochemistry**. [S.l.] : Treatise on Geochemistry, 2003. p. 149–204.

BORKOVIĆ-MITIĆ, Slavica S. et al. - Biomarkers of oxidative stress and metal accumulation in marsh frog (*Pelophylax ridibundus*). **Environmental Science and Pollution Research**. ISSN 16147499. 23:10 (2016) 9649–9659. doi: 10.1007/s11356-016-6194-3.

BURGER, J.; SNODGRASS, J. - Metal levels in Southern Leopard Frogs from the Savannah river site: location and body compartment effects. **Environmental Research.** 86:2 (2001) 157–166. doi: 10.1006/enrs.2001.4245.

CAREY, Cynthia; BRYANT, CJ - Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. **Environmental Health Perspectives**. 1995) 13–17.

COSTA, S. *et al.* - Diversity of cutaneous bacterial community of *Pelophylax perezi* populations inhabiting different environments. **Science of the Total Environment**. ISSN 1098-6596. in press:2016). doi: 10.1016/j.scitotenv.2016.07.230.

DI TORO DM, Allen HE, Bergman HL, Meyer JS, Paquin PR, Santore RC. (2001). Biotic ligand model of the acute toxicity of metals. 1. Technical basis. **Environ Toxicol Chem** 20: 2383–2396. DOBROVOLJC, Katarina et al. - Cd, Cu, Zn, Se, and metallothioneins in two amphibians, *Necturus maculosus* (Amphibia, Caudata) and *Bufo bufo* (Amphibia, Anura). **Biological trace element research**. . ISSN 1559-0720. 150:1–3 (2012) 178–94. doi: 10.1007/s12011-012-9461-2

DOBSON, Alleson et al. - Bacteriocin production: A probiotic trait? **Applied and Environmental Microbiology**. ISSN 00992240. 78:1 (2012) 1–6. doi: 10.1128/AEM.05576-11.

EDMONDSON, J. C. - Mining in the later Roman Empire. **The Journal of Roman Studies**. 79:1989) 84–102.

FASOLA, E.; RIBEIRO, R.; LOPES, I. - Microevolution due to pollution in amphibians: A review on the genetic erosion hypothesis. **Environmental Pollution**. ISSN 02697491. 204:2015) 181–190. doi: 10.1016/j.envpol.2015.04.027.

HARRIS, RN; BRUCKER, RM; WALKE, JB - Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. **The ISME Journal** . ISSN 1751-7362. 3:7 (2009) 818–824. doi: 10.1038/ismej.2009.27.

HIGUERAS, P. *et al.* - The Almadén district (Spain): anatomy of one of the world's largest Hg-contaminated sites. **Science of the Total Environment**. ISSN 00489697. 356:1–3 (2006) 112–124. doi: 10.1016/j.scitotenv.2005.04.042.

HOFFMANN, A. A.; PARSONS, P. A. - **Evolutionary genetics and environmental stress.** Oxford, UK ed. [S.l.] : Oxford University Press, 1991

HORNE, M. T.; DUNSON, W. A. - Effects of low pH , metals , and water hardness on larval amphibians. **Arch. Environ. Contam. Toxicol.** 29 (1995) 500–505.

JOFRÉ, MB; ANTÓN, RI; CAVIEDES-VIDAL, E. - Lead and cadmium accumulation in anuran amphibians of a permanent water body in arid Midwestern Argentina. **Environmental Science and Pollution Research.** (2012) 2889–2897. doi: 10.1007/s11356-012-0795-2.

LI, Hong; OTVOS, James D. - 111Cd NMR studies of the domain specificity of Ag+ and Cu+ binding to metallothionein. **Biochemistry**. ISSN 00062960. 35:44 (1996) 13929–13936. doi: 10.1021/bi961401n.

LINDBERG, S. E. *et al.* - Atmospheric emission and plant uptake of mercury from agricultural soils near the Almadén mercury mine. **Journal of Environmental Quality**. 8:4 (1978) 572–578. doi: 10.2134/jeq1979.00472425000800040026x.

MACNAIR, M. N. - Why the evolution of resistance to anthropogenic toxins normally involves major gene changes: The limits to natural selection. **Genetica**. 84 (1991) 213–219.

MARQUES, Sérgio M. et al. - Antioxidant response and metal accumulation in tissues of Iberian green frogs (*Pelophylax perezi*) inhabiting a deactivated uranium mine. **Ecotoxicology** (London, England). ISSN 1573-3017. 20:6 (2011) 1315–27. doi: 10.1007/s10646-011-0688-z.

MARTINS, Nelson *et al.* - Effects of acid mine drainage on the genetic diversity and structure of a natural population of *Daphnia longispina*. **Aquatic Toxicology**. . ISSN 1879-1514. 92:2 (2009) 104–112. doi: 10.1016/j.aquatox.2009.01.007.

MCKENZIE, V. J. *et al.* - Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. **ISME**. 2012) 588–596. doi: 10.1038/ismej.2011.129.

MEDINA, MH; CORREA, JA; BARATA, Carlos - Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. **Chemosphere**. 67:2007) 2105–2114. doi: 10.1016/j.chemosphere.2006.12.024.

NORDSTROM, D. K.; ALPERS, C. N. - Geochemistry of acid mine waters. Em PLUMLEE, G. S.; LOGSDON, M. J. (Eds.) - **The Environmental Geochemistry of Mineral Deposits.** [S.l.] : Reviews in Economic Geology 6A, 1999. p. 133–160.

O'BRIEN, John; ALLENTOFT, Morten E. - Global Amphibian Declines, Loss of Genetic Diversity and Fitness: A Review. **Diversity**. ISSN 1424-2818. 2:1 (2010) 47–71. doi: 10.3390/d2010047.

PASK, JD - The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. **Global Change** (2012) 1231–1238. doi: 10.1111/j.1365-2486.2011.02622.x.

PEREIRA, Ruth; RIBEIRO, Rui; GONÇALVES, Fernando - Plan for an integrated human and environmental risk assessment in S. Domingos Mine area (Portugal). **Human and Ecological Risk Assessment: An International Journal**. 10:2004) 543–578.

REGLERO, M. *et al.* - Transfer of metals to plants and red deer in an old lead mining area in Spain. **Science of the Total Environment**. ISSN 00489697. 406:1–2 (2008) 287–297. doi: 10.1016/j.scitotenv.2008.06.001.

RIBEIRO, R; LOPES, I- Contaminant driven genetic erosion and associated hypotheses on alleles loss, reduced population growth rate and increased susceptibility to future stressors: an essay. **Ecotoxicology (London, England)**. ISSN 1573-3017. 22:5 (2013) 889–99. doi: 10.1007/s10646-013-1070-0.

SKRODENTYE-ARBACIAUSKIENE V, SROUGA A, BUTKAUSKAS D. (2006). Assessment of microbial diversity in the river trout Salmo trutta fario L. intestinal tract identified by partial 16S rRNA gene sequence analysis. **Fish Sci** 72: 597–602.

SMITH, M. Alex; GREEN, David M. - Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? **Ecography**. 28:1 (2005) 110–128. SOBRAL, Olímpia; MARIN-MORALES, Maria Aparecida; RIBEIRO, Rui - Could contaminant induced mutations lead to a genetic diversity overestimation? **Ecotoxicology**. ISSN 09639292. 22:5 (2013) 838–846. doi: 10.1007/s10646-013-1079-4.

SPARLING, D. W.; T.P., Lowe. - Metal concentrations of tadpoles in experimental ponds. **Evironmental pollution**. 91:2 (1996) 149–159. doi: 10.1016/0269-7491(95)00057-7.

SPARLING, Donald W.; KREST, Sherry; ORTIZ-SANTALIESTRA, Manuel - Effects of lead-contaminated sediment on *Rana sphenocephala* tadpoles. **Archives of Environmental Contamination and Toxicology**. ISSN 00904341. 51:3 (2006) 458–466. doi: 10.1007/s00244-005-0243-0.

SPARLING, DW et al. - Ecotoxicology of amphibians and reptiles. 2010).

STILLMAN, M. J.; ZELAZOWSKI, A J. - Domain-specificity of Cd2+ and Zn2+ binding to rabbit liver metallothionein. Metal ion mobility in the formation of Cd4-metallothionein alpha-fragment. **The Biochemical journal**. ISSN 0264-6021. 262:1 (1989) 181–8.

TAYLOR, D. H.; STEELE, C. W.; STRICKLER-SHAW, S. - Responses of green frog (*Rana Clamitans*) tadpoles to lead-polluted water. **Environmental Toxicology and Chemistry**. 9:1 (1990) 87–93. doi: 10.1002/etc.5620090111. doi: 10.1897/1551-5028(2001)020<2383:blmota>2.0.co;2.

TIMMERMANS, Martijn J. T. N. *et al.* - Metallothionein mRNA expression and cadmium tolerance in metal-stressed and reference populations of the springtail *Orchesella cincta*. **Ecotoxicology**. ISSN 09639292. 14:7 (2005) 727–739. doi: 10.1007/s10646-005-0020-x.

VAN STRAALEN N.M.; TIMMERMANS M.J.T.N. - Genetic variation in toxicants-stressed populations: An evaluation of the «Genatic Erosion» Hypothesis (2002).

VERTUCCI, F. A.; CORN, P. S. - Evaluation of episodic acidification and amphibian declines in the Rocky Mountains. **Ecological Applications**. 6:1996) 449–457.

WANG, Mei Zhen; JIA, Xiu Ying - Low levels of lead exposure induce oxidative damage and DNA damage in the testes of the frog Rana nigromaculata. **Ecotoxicology**. ISSN 09639292. 18:1 (2009) 94–99. doi: 10.1007/s10646-008-0262-5.

WOODHAMS, Douglas C. e*t al.* - Managing Amphibian Disease with Skin Microbiota. **Trends in Microbiology**. . ISSN *0966842X. 24:3 (2016) 161–164. doi: 10.1016/j.tim.2015.12.010.*

WOLF, Arite *et al*. - *Stenotrophomonas rhizophila* sp. nov., a novel plant-associated bacterium with antifungal properties. **International Journal of Systematic and Evolutionary Microbiology**. ISSN 14665026. 52:6 (2002) 1937–1944. doi: 10.1099/ijs.0.02135-0.